**University of Alberta**

# A new tissue model for evaluating effects of *Bacillus thuringiensis* toxins on insect midgut epithelium.

by

Lorraine Braun

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Department of Entomology

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**University of Alberta**

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**Dedication**

This thesis is dedicated with love to my partner, friend and husband Ross J. Peters. He left behind friends and family to accompany me in this quest for knowledge and I appreciate that more than I can say. I thank him for his emotional support, patience, and endless encouragement.

**Abstract**

Current methods used to determine efficacy of *Bacillus thuringiensis* (B.t.) δ-endotoxins in Lepidoptera utilize feeding bioassays, histological studies of midgut epithelium, cultured insect cells and production of brush border membrane vesicles. Presented here is a new model which uses living lepidopteran midgut epithelium wholemounts produced after enzymatic removal of basal lamina and connective tissue from midguts of *Trichoplusia ni* larvae. Wholemounts were nourished in artificial haemolymph and tissue viability was assessed for up to 24 h using the vital dyes trypan blue, acridine orange (AO), propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI). Peritrophic membrane and basal lamina synthesis and modification of B.t. Cry1Ac protoxin to active toxin demonstrated some normal epithelial function. Enzymatic removal of basal lamina and connective tissue did not result in loss of midgut tissue viability or increased cell staining by trypan blue. Likewise short exposure to UV radiation, and addition of solubilization buffer and *E. coli* cell products did not increase cell membrane permeability. Descriptions of cells and midgut tissue from untreated, healthy insects provide a basis for comparison after tissues are treated with B.t. toxins. Treatment of tissues with solubilized Cry1Ac protoxin resulted in increased membrane permeability to vital dyes in columnar epithelial and regenerative cells; feeding and oral inoculation bioassays verified Cry1Ac toxicity *in vivo*.

DAPI was selected to identify cells targeted by the single-gene products Cry1Ab, Cry1B, Cry1C, Cry1E, Cry2A and Cry3A. Different B.t. toxins caused different DAPI staining patterns in epithelial wholemounts and reactions of toxins with a high degree

of amino acid homology (Cry1Ac, Cry1Ab, Cry1E) were similar. The coleopteran-specific Cry3A did not increase cell membrane permeability. Staining in columnar and regenerative cells was correlated with *in vivo* toxicity.

Effects of B.t. δ-endotoxins can be examined directly in midgut epithelial cells and whole tissue response can be observed. This epithelial tissue model provides a rapid and sensitive assay and should prove useful in monitoring development of resistance to B.t. toxins, in assessing effects on non-target Lepidoptera, and in further research into mode of action at the cellular level.

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**List of abbreviations and acronyms**

| | |
|---|---|
| AO | acridine orange |
| ATP | adenosine 5'-triphosphate |
| ATPase | adenosine triphosphatase (an enzyme that catalyzes hydrolysis of ATP) |
| bas | small basal cell |
| BBMV | brush border membrane vesicle |
| BP | band pass |
| BSA | bovine serum albumin |
| B.t. | *Bacillus thuringiensis* |
| B.t.k. | *Bacillus thuringiensis* var. *kurstaki* |
| col | columnar epithelial cell |
| DAPI | 4',6-diamidino-2-phenylindole |
| DNA | deoxyribonucleic acid |
| DTT | dithiothreitol |
| DYLT | daylight |
| edg | dead or damaged cells along cut edges |
| ext | extruding columnar epithelial cell |
| GPI | glycosyl-phosphatidylinositol |
| gob | goblet cell |
| 20-HT | 20-hydroxyecdysone |
| ICP | insecticidal crystal protein |
| IPM | integrated pest management |
| kD | kilodalton |
| $LC_{50}$ | median lethal concentration |
| $LD_{50}$ | median lethal dose |
| min | minute(s) |
| nd | not done |
| np | could not prepare wholemount |
| PI | propidium iodide |
| PIPLC | phosphatidylinositol-specific phospholipase C |
| PM | peritrophic membrane |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |

| | |
|---|---|
| SAHS | sterile artificial haemolymph solution |
| SD | standard deviation |
| SDS-PAGE | sodium dodecyl sulfate - polyacrylamide gel electrophoresis |
| td | overall tissue damage |
| U1 | ultraviolet filter, band pass 330-380 |
| UV | ultraviolet |
| V-ATPase | vacuolar ATPase |

## Chapter 1 Introduction to *Bacillus thuringiensis*

### 1.1 Early history of *Bacillus thuringiensis*

In 1901, Ishiwata identified the causal agent of sotto disease of silkworms, *Bombyx mori*, as a spore-forming bacillus which he named "sotto disease bacillus" (Heimpel and Angus, 1958). Berliner isolated the same pathogen from diseased Mediterranean flour moths, *Anagasta kühniella*, and in 1911 named it *Bacillus thuringiensis* (B.t.). He described the bacteria as a gram-positive spore-forming rod with a "Restkörper" or remaining body formed during sporulation (Berliner, 1915 cited in Hannay and Fitz-James, 1955). Although the first field test using B.t. was performed against European corn borer, *Ostrinia nubilalis*, in 1929 (Husz 1929, cited in Tanada and Kaya, 1993) B.t.'s potential as an insect control agent was not fully appreciated until work on it resumed at the University of California. In 1942 E. Steinhaus obtained a culture of *Bacillus thuringiensis* Berliner from N. Smith, who had received it from J. R. Porter in 1940 (Steinhaus, 1951). Porter had obtained the culture from O. Mattes, who had reisolated it from diseased larvae of Mediterranean flour moth. Steinhaus also determined that three strains of *Bacillus cereus* isolated from diseased *Plodia, Aphomia* and *Ephestia* were pathogenic for insects (Steinhaus, 1951). These three strains were unlike typical *B. cereus* in that they all contained spores positioned obliquely to the sporangial wall, and were therefore reclassified as *B. thuringiensis* (Steinhaus, 1951).

In Canada, research was focused on determining the exact nature of B.t.'s toxicity. C.L. Hannay (1953) redescribed the Restkörper as a diamond-shaped crystal which formed in the bacterial cell at the opposite end from the spore, and Hannay and Fitz-

James (1955) showed that crystals were proteinaceous, and composed of at least 17 amino acids. In 1954 T. Angus demonstrated that an insecticidal extract is obtained when crystals are treated with clarified silkworm gut juice at pH 9.5 - 10 (Angus, 1954). Insects injected with this extract died from septicaemia; insects fed the extract suffered first from paralysis then septicaemia. Heimpel and Angus (1959), using histological techniques, determined that the site of action of the toxic crystal protein is the midgut epithelium. They then devised a scheme to classify Lepidoptera based on their response to B.t.: type I species exhibit gut and general paralysis associated with a blood pH change after ingesting crystals; type II insects stop feeding after ingesting crystals, suffer from gut paralysis without an associated blood pH change, and die several days later; and type III larvae die after ingesting a spore-crystal mixture without exhibiting general paralysis. They noted that classification varies with dosage, and that intermediates between types I and II exist. Type IV insects were described by Martouret (1961, cited in Heimpel, 1967) as Lepidoptera (such as some noctuids) not susceptible to crystal toxins.

Basic research on B.t. continued (*i.e.* genetic characterization, strain identification, and serotyping) (De Barjac and Bonnefoi, 1962; Dulmage, 1970; Goldberg and Margalit, 1977; González and Carlton, 1980). Meanwhile increasing public pressure to find alternatives to chemical insecticides and the development of insect resistance to insecticides has lead to an explosive increase in research on production of agricultural products based on B.t. Unfortunately research on mode of action has lagged behind research on cloning B.t. (Schnepf and Whiteley, 1981) and development of transgenic plants (Vaeck *et al.*, 1987; Fischoff *et al.*, 1987; Barton *et al.*, 1987) occurred before it

was determined exactly how B.t. killed insects. Research in recent years has focused on mode of action and receptor binding, while identification and genetic engineering of novel toxins to use on agricultural pests continues. There is still very little known on the ecology of B.t. or its effect on non-target invertebrates (Addison, 1993).

## 1.2 Distribution of B.t.

*Bacillus thuringiensis* is a gram-positive soil bacterium with worldwide distribution (Martin and Travers, 1989; Martin, 1994). Although it has been isolated in soil samples from tropical jungles, beaches, deserts, arctic tundra, mountains, temperate and tropical caves, agricultural fields, grasslands, scrub wilderness and urban environments, Martin and Travers (1989) had more success isolating high numbers of B.t. isolates from environments with no detectable insects than from soils with high insect activity (fire ants). Of isolates recovered, most are lepidopteran-specific although a large percentage (up to 64%) show no toxicity to any insect tested (Ohba and Aizawa, 1986; Martin and Travers, 1989; Smith and Couche, 1991; Meadows *et al.*, 1992; Chilcott and Wigley, 1993). These figures partially reflect methods used and the range of insects tested. Numbers of nontoxic isolates might decrease if all isolates were tested against several lepidopteran, dipteran and coleopteran species. Ohba and Aizawa (1986) suggest that nontoxic strains are distributed more widely in the environment than toxic ones. These nontoxic isolates may also play an as yet undiscovered role in the environment (Martin, 1994).

Strains of B.t. have also been recovered from the phylloplane of temperate trees and shrubs, in frequencies higher than expected from casual isolations (50 - 70% of trees

were positive for B.t.) (Smith and Couche, 1991). The majority of isolates (63%) were active against lepidopteran larvae, with fewer (16%) active against coleopteran larvae. These isolates did not result from previous commercial applications because some did not respond to antibodies reactive against proteins from the three pathotypes formulated into commercial products, and there were isolates specific to Coleoptera. Several (5%) were apparently novel combinations of toxin genes, active against both lepidopteran and coleopteran larvae.

### 1.3 Ecology of B.t.

The ecological role of B.t. in the soil is poorly understood (Addison, 1993; Martin, 1994). Although it is ubiquitous in the environment, B.t. is not known to cause epizootics in nature (epizootics do occur in man-made enclosed environments such as grain storage bins) (DeLucca *et al.*, 1981). Persistence of B.t. in some environments, such as abandoned grain storage bins and in soils near mass-rearing facilities lasts several years (Addison, 1993; DeLucca *et al.*, 1981, DeLucca *et al.*, 1982) although B.t. is not considered a soil saprophyte (B.t. is unable to compete with *B. cereus* for a similar niche in soil so it is unlikely that it is an active constituent in the soil microbial community) (Addison, 1993). B.t. likely exists in two separate ecologies which seldom interact: natural environments and stored-product environments (DeLucca *et al.*, 1982). B.t. is thought to exist in the soil as an inactive spore, unable to germinate and grow saprophytically except under specialized circumstances (Addison, 1993). Although crystal proteins released from B.t. cells into the soil are degraded quickly, spores are long-lived (about one year) (Lambert and Peferoen, 1992). B.t. has the capacity to

produce either rapidly or slowly germinating spores in the absence or presence of a crystal (Stahly *et al.*, 1978). This unique ability may confer flexibility for germination and growth in the soil environment, since survival outside an insect host in the more competitive soil environment may be dependent on the ability of spores to germinate rapidly. Spores themselves do have some toxicity, but very high numbers are required to kill insects, and since B.t. spore numbers in soil are relatively low, they likely do not directly regulate insect populations in nature (Lambert and Peferoen, 1992). Daily collections from grassy sites showed daily population fluctuations, with overall numbers highest in spring and fall and lowest in summer (Martin, 1994). Martin (1994) showed that B.t. dynamics in soil consisted of rapid daily multiplication with gradual changes in population density and phenotypic diversity over time.

If B.t. is strictly an insect pathogen it should be found in the same environment as its host insect, where it should occasionally cause epizootics (Martin, 1994). Spore isolations from these sites should be dominated by one species, however soil isolates containing species of B.t. toxic to only a single insect species are rare; most samples have mixed toxicity types. And it has been shown that B.t. has a broad environmental distribution unrelated to its target insects (Martin, 1994). B.t. is not an obligate pathogen since it grows, sporulates and produces toxic crystals on a wide variety of artificial media, at temperatures and pHs considered unsuitable for insects (10 - 15°C and pH 5 - 6). In most cases germination and growth of B.t. occur in the insect before death, with conditions quickly becoming anaerobic and unfavourable for B.t. replication and sporulation when the insect dies (Lüthy and Ebersold, 1981; Martin, 1994).

B.t. is a soil bacterium producing parasporal crystals with insecticidal properties at considerable metabolic cost to the cell through its increased genome and synthesis of protein during the nutrient stress of sporulation (Martin, 1994). The rate of toxin protein synthesis is expensive, 33-43% of the overall rate of protein synthesis, so it must confer a selective advantage to the bacteria (Feitelson *et al.*, 1992). If these crystals do not affect insects in the environment where B.t. is found (Addison, 1993), it is possible they do regulate other soil invertebrates, such as nematodes (Lambert and Peferoen, 1992). Strains of B.t. active against zooparasitic nematodes (mainly larvicidal and ovicidal effects), earthworms, some ants, predacious phytoseiid mites (affect egg production and larval development) and Collembola have been identified (Addison, 1993). Introduction to soil of a high inoculum or new genetically-engineered strains may upset natural ecological relationships and may be detrimental to beneficial soil invertebrates.

Ecological relationships among B.t., plants, and insects are not well understood, although B.t. isolates may act as larval antifeedants, providing plant protection against insects while deriving some benefit from the plant (Smith and Couche, 1991). The environmental half-life of B.t. applied to plants is only about one day (Martin, 1994), but there is some evidence that B.t. applications would likely disrupt naturally occurring *Bacillus* species, including B.t., already present. Application of a strain toxic to Colorado potato beetle resulted in disappearance of any original *Bacillus* strains present on potato, tomato, eggplant and green pepper plants (Martin, 1994). Counts of *Bacillus* strains decreased steadily post-treatment although total bacterial counts remained steady, indicating that non-*Bacillus* strains replaced both original strains and applied strains.

## 1.4 Classification of B.t.

Taxonomy of *B. thuringiensis* is complex: based on DNA sequence homology studies it is placed in a phenetic group containing *Bacillus anthracis, B. cereus* and *Bacillus mycoides* (50-100% homology) (Somerville and Jones, 1972), however *B. thuringiensis* and *B. anthracis* are traditionally designated as separate species based on their important pathogenic attributes (Sneath *et al.*, 1986). *Bacillus cereus* strains are scattered throughout dendrograms prepared from rRNA gene restriction patterns (43 *B. thuringiensis* isolates) and phenotypic analysis whereas both *B. anthracis* strains and *Bacillus sphaericus* strains are readily distinguished (Baumann *et al.*, 1984; Priest *et al.*, 1994). Comparison of phenotypic characteristics of *B. thuringiensis* and *B. cereus* (137 strains of *B. thuringiensis* and 35 strains of *B. cereus*) has shown that the two species are undistinguishable except for their ability to produce parasporal crystals (Baumann *et al.*, 1984). The ease with which plasmids are transferred from *B. thuringiensis* strains to *B. cereus* (Battisti *et al.*, 1985; González *et al.*, 1982) and *B. anthracis* (Battisti *et al.*, 1985) supports this close relationship. Since many *B. thuringiensis* stock strains in culture lose their plasmids and therefore their toxicity to insects, some authors (Gordon, 1976; Baumann *et al.*, 1984) feel that they should only be variants of *B. cereus*, whereas Martin (1994) states that *B. cereus* isolates may be only degenerate forms of *B. thuringiensis*. Although *B. thuringiensis* is genetically heterogeneous, as indicated by both ribotyping and DNA/DNA hybridization (Somerville and Jones, 1972), there is a strong correlation between ribotype and serotype, indicating limited chromosomal gene exchange during evolution (Priest *et al.*, 1994).

One explanation for the enormous diversity of B.t. toxins is that genes for toxic proteins are usually encoded on conjugative plasmids (Höfte and Whiteley, 1989), and there is opportunity for gene transfer through conjugation within insect hosts or in the environment. Members of the *Bacillus* genus are also capable of natural transformation, so free DNA in the soil or aquatic environment can be incorporated into the genome (Lorenz and Wackernagel, 1994). Diversity may arise also from coevolution of bacterial genes that encode toxin proteins, and toxin-target genes in the host (Feitelson *et al.*, 1992). The number and type of individual genes and their total protoxin concentration in the crystals presumably determines the overall host range of the individual B.t. strain (Brousseau and Masson, 1988).

Initial classification of B.t. strains, based on serotyping of flagellar antigens and biochemical tests, resulted in fifteen serotype groups (de Barjac, 1981). Within ten years the number had risen to thirty-four serovars including twenty-seven antigenic groups (de Barjac and Frachon, 1990). B.t. crystal protein genes are classified according to the type of insect they are active against: cryI - Lepidoptera; cryII - Lepidoptera and Diptera; cryIII - Coleoptera; cryIV - Diptera; cryV - Lepidoptera and Coleoptera; and cry VI - Nematoda (Höfte and Whiteley, 1989; Feitelson *et al.*, 1992). Within each class of toxin there are several subclasses (*e.g.* cryIA(a), cryIB etc.). This nomenclature and classification scheme, based on both amino acid sequence of crystal proteins and host range, became unworkable when it was found that genes with similar sequences differed in host specificity. Ninety-six different crystal protein genes have now been sequenced, encompassing seventeen different homology groups (cry1 to cry15 plus 2 cyt genes), and

the proposed revised nomenclature scheme is based solely on amino acid identity (Crickmore *et al.*, 1995). (In this scheme Roman numerals have been replaced by Arabic numerals *i.e.* CryIA(c) = Cry1Ac.)

### 1.5 Toxins produced by B.t.

Heimpel (1967) reviewed the various toxins produced by B.t. strains and proposed a scheme of nomenclature: α-exotoxin (previously referred to as phospholipase C); β-exotoxin (previously called the fly factor or the thermostable exotoxin); γ-exotoxin (egg yolk clearing factor); and δ-endotoxin, the crystalline parasporal body. This scheme was later modified by Krieg and Lysenko (1979, cited in Tanada and Kaya, 1993). The designation of γ-exotoxin, or yolk-clearing factor, was discontinued because it is not toxic to insects (Tanada and Kaya, 1993).

α-exotoxin is a proteinaceous thermo-, pH-, and trypsin-sensitive exotoxin which causes degeneration and lysis of haemocytes in insects and is also toxic to mice (Krieg, 1971). Phospholipase C or lecithinase C, is a thermolabile enzyme produced by some subspecies of B.t. and *B. cereus* which is toxic to insects (Heimpel, 1967).

β-exotoxin, or thuringiensin, is a heat-stable toxin produced by some B.t. strains (Ohba *et al.*, 1981) which affects a broad range of insects, other invertebrates and vertebrates (Tanada and Kaya, 1993; Lecadet and de Barjac, 1981). It is an ADP analog which specifically inhibits DNA-dependent RNA polymerase. In insects it causes larval death, deformed pupae, moulting problems or abnormal adults. Because it is toxic to vertebrates, most commercial formulations of B.t. use species that do not produce β-exotoxin.

δ-endotoxins are a class of toxic proteins produced from crystal proteins or protoxins that are degraded by proteolytic enzymes to form smaller toxic peptides, the endotoxins (Tanada and Kaya, 1993). The crystals are produced during sporulation and released from the bacterial cell with the spore after cell lysis (the spore coat may also contain quantities of the crystal protein). They are denatured by heat, and are insoluble in water and organic solvents, and soluble in highly alkaline conditions. At lower pH a reducing agent is required to help dissolve the crystal. Crystals are of several shapes and are composed of one to many different proteins encoded by different crystal protein genes. Crystals have been found that are toxic to several insect orders, plant and animal-parasitic nematodes, protozoan pathogens, and animal-parasitic liver flukes and mites (Feitelson *et al.*, 1992).

### 1.6 Mode of action of B.t. δ-endotoxin

Several excellent, recent reviews have described mode of action studies and crystal protein structure (Knowles, 1994; Li *et al.*, 1991; Grochulski *et al.*, 1995). Effects of B.t. on Lepidoptera have been determined through feeding bioassays and studies using histology, brush border membrane vesicles (BBMVs) and cultured cell lines. Parasporal crystal proteins are solubilized in the alkaline midgut of susceptible insects to form protoxin; in insects with lower midgut pH, reducing agents are required to dissolve the crystal. Protoxin is then converted to an active form by midgut proteases which cleave it down to a smaller protease-resistant core. In lepidopteran-specific 130-145 kD protoxins, the activated toxin resides in the large 62-70 kD N-terminal portion of the protoxin. Susceptible insects display paralysis of midgut and mouthparts resulting in feeding

inhibition within 5 min after ingestion of B.t. δ-endotoxin (Hannay, 1953; Angus, 1954).

### 1.6.1 Cellular effects of lepidopteran-specific δ-endotoxins

Studies with BBMVs (Tojo, 1986; Hofmann *et al.*, 1988a; Hofmann *et al.*, 1988b; Van Rie *et al.*, 1989; Van Rie *et al.*, 1990a) have confirmed observations from early (Heimpel and Angus, 1960; Fast and Angus, 1965; Ebersold *et al.*, 1977) and more recent histological studies (Oron *et al.*, 1985; Lane *et al.*, 1989): toxin receptors are located on the apical membrane of midgut epithelial cells. Ultrastructural changes occur in columnar epithelial cells within a few minutes of ingestion of B.t. crystals. Cytological effects include enlargement of rough endoplasmic reticulum cisternae, swelling of mitochondria and Golgi complexes, disruption of the microvillar border and deformation of the basal epithelium (Ebersold *et al.*, 1977; Endo and Nishiitsutsuji-Uwo, 1981; Percy and Fast, 1983; Oron *et al.*, 1985; Lane *et al.*, 1989; Mathavan *et al.*, 1989). Disruption of ion regulation in cell membranes leads to inhibition of the $K^+$ pump in goblet cells (Gupta *et al.*, 1985), which causes a dramatic increase of $K^+$ and $H^+$ in the haemolymph, and an increase of haemolymph pH with a decrease in midgut pH (Heimpel and Angus, 1959; Nishiitsutsuji-Uwo and Endo, 1980). B.t. toxins also stimulate glucose uptake (Fast and Donaghue, 1971) and decrease ATP concentration in midgut cells (Travers *et al.*, 1976). Alterations in columnar cell permeability lead to cell swelling, exfoliation, and eventual disintegration of the epithelium (Cooksey, 1971).

### 1.6.2 Structure of δ-endotoxin and pore formation

The tertiary structure of Cry3A has been determined by x-ray crystallography (Li *et al.*, 1991). The protein has three structurally distinct domains: domain I consists of the

N-terminal 290 amino acid residues formed into a cluster of seven ∝-helices; domain II, including residues 291 through 500, is composed of three structurally homologous β-sheets; domain III, extending to the C-terminus, is composed of several anti-parallel strands (Yamamoto and Powell, 1993). It is hypothesized that domains II and III determine insect specificity and receptor binding, while domain I is responsible for pore formation. In a current model, domain II binds to a membrane protein receptor. The toxin molecule swings open on a hinge linking domains I with domains II and III. Domain I then inserts into the membrane creating a pore (domain I may form polymers *e.g.* hexamers which create a 20 Å pore) (Knowles, 1994).

### 1.6.3 B.t. toxin receptors

Several theories have been proposed to explain the mode of action of δ-endotoxins (Appendix 1) but as yet it is not known if: the receptor is an existing channel opened by toxin; toxin combines with a receptor to form a new channel; or the receptor is a membrane protein which assists toxin insertion into the membrane. A two-step model by Knowles and Ellar (1987) states that the toxin binds specifically to a cell surface receptor and generates small pores in the membrane. Equilibration of ions through these pores results in a net influx of ions accompanied by water which causes swelling and lysis (the colloid osmotic lysis theory).

Binding experiments using BBMVs and midgut tissue sections have shown that there are at least two different receptors for B.t. insecticidal crystal proteins (ICPs) in midgut epithelium of *Ostrinia nubilalis* (Denolf *et al.*, 1993). Cry1Ab and Cry1Ac share the same receptor while Cry1B has a different receptor. Van Rie *et al.* (1989) showed

that in both *Manduca sexta* and *Heliothis virescens* Cry1Aa, Cry1Ab, and Cry1Ac share one receptor, while Cry1Ab and Cry1Ac share a second receptor, and Cry1Ac also binds to a third receptor. Using BBMVs, Hofmann *et al.* (1988a) showed that there are two distinct binding sites for Cry1Ac and Cry1B in *Pieris brassicae*. In a resistant strain of *Plutella xylostella*, Cry1C recognizes a different receptor than Cry1Ab and Cry1B (Ferré *et al.*, 1991). Likewise Estada and Ferré (1994) showed that Cry1Aa did not compete with Cry1Ab or Cry1Ac for the same binding site in B.t.-resistant *Trichoplusia ni*.

Aminopeptidase N, a 120-kD glycoprotein, has been identified as the receptor for Cry1Ac in *M. sexta* (Knight *et al.*, 1994; Sangadala *et al.*, 1994) and *Lymantria dispar* (Valaitis *et al.*, 1995). It is anchored in the membrane of epithelial cells of *M. sexta* by glycosyl-phosphatidylinositol (GPI) (Garczynski and Adang, 1995; Knight *et al.*, 1995).

### 1.7 Resistance to B.t.

Specificity among isolates is due to some or all of the following: affinity of binding sites on midgut epithelium; differential proteolytic processing within the larval gut; variation in peptide sequence of the toxin; and variation in the number of toxic peptides. Resistance can arise from behaviourial changes (insects avoid eating toxins) or physiological changes (gut pH, numbers of receptors, changes to enzymes which affect toxin dissolution and activation).

Laboratory selection for resistance to B.t. δ-endotoxin has been achieved in Lepidoptera (*Plodia interpunctella, Cadra cautella, Plutella xylostella, Heliothis virescens, Homeosoma electellum, T. ni*) (McGaughey, 1985; McGaughey and Beeman, 1988; Stone *et al.*, 1989; Tabashnik *et al.*, 1991; Brewer, 1991; Estada and Ferré, 1994),

Coleoptera (*Leptinotarsa decemlineata*) (Whalon *et al.*, 1993), and Diptera (*Culex quinquefasciatus*, *Aedes aegypti*) (Goldman *et al.*, 1986). Diamondback moth is the only insect reported to have high levels of resistance in the field, and this has been observed in Hawaii (Tabashnik *et al.*, 1990) and the Philippines (Whalon and McGaughey, 1993).

Studies on the biochemical basis of resistance have shown that resistance occurs through different mechanisms in different insects (Estada and Ferré, 1994). For example, resistance of *H. virescens* to Cry1Ac is not accompanied by significant changes in toxin binding or binding site concentrations (Gould *et al.*, 1992), or alteration of toxin by midgut proteases (Lee *et al.*, 1995). In *Plodia interpunctella*, resistance to Cry1Ab is due to an alteration in toxin-membrane binding (Van Rie *et al.*, 1990b). Again, midgut proteinases are not involved in this resistance mechanism: resistant and susceptible strains are similar in proteolytic activity and ability to activate B.t. protoxin (Johnson *et al.*, 1990). Dent (1991) warns that laboratory-based studies of resistance inheritance may have little relationship to field-induced mechanisms of resistance because polygenic inheritance often occurs in laboratory selection experiments whereas changes in major genes occur more commonly in the field.

Development of cross-resistance must be considered in all management strategies. Tabashnik *et al.* (1994) found cross-resistance to Cry1F when *P. xylostella* was selected with Cry1A and Cry2 toxins, however only minimal cross-resistance to *B. thuringiensis* subsp. *aizawai* developed in insects selected against *B. thuringiensis* subsp. *kurstaki* (Tabashnik *et al.*, 1993). Also, a selected line of cabbage looper, *T. ni*, developed resistance only to the ICP for which it was selected, Cry1Ab, and not to the closely

related toxins Cry1Aa or Cry1Ac (Estada and Ferré, 1994). Development of cross-resistance is less likely if toxins recognize different receptors, and use of ICP mixtures or multiple ICP expressing transgenic plants would likely be a valuable resistance management tactic (Van Rie *et al.*, 1992).

Strategies recommended to manage resistance to B.t. are generally patterned after those used to manage chemical insecticide resistance and include rotation or alteration of toxins, toxin mixtures, establishment of refugia, differential dosage, and B.t. gene regulation in transgenic plants (Whalon and McGaughey, 1993). Rotation or alteration of B.t. toxins with other insecticides, and cultural or biological control practices are the approaches most often used with chemical insecticides. Success depends on restoring susceptibility when selection pressure is discontinued. Toxin mixtures or sequences can be used with both conventional delivery methods and transgenic plants, however development of cross-resistance must be monitored carefully. Provision of refugia or immigration of susceptible insects probably provide the best resistance strategy but require extensive research to determine optimum spatial and temporal scales. Low toxin doses work well when pest levels are below economic injury levels, but they do not prevent damage. High toxin expression in transgenic plants should kill most heterozygotes[1] and resistance development would be delayed if untreated refuges supplied susceptible individuals to mate with survivors. Alteration of tissue location, timing, or induction of B.t. toxin expression in transgenic plants may also reduce

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[1] High dose is usually defined as the dose which consistently kills heterozygotes (McGaughey and Whalon, 1992). The toxin dose required is lowest when the resistance trait is recessive and highest when the trait is dominant.

selection pressure. Resistance monitoring programs and integration of resistance management into IPM programs are essential for most of these approaches.

Transgenic plants which express toxins directly remove requirements for specific gut conditions which activate protoxins, and could potentially expand the range of non-target hosts (Addison, 1993). In addition transformed plants containing single or multiple δ-endotoxins do not have the advantage of the whole bacterial organism: they lack spores and other B.t. toxins which contribute to insect mortality, including β-exotoxin (thuringiensin).

### 1.8 Use of B.t. as a bioinsecticide

B.t. is currently the insecticide of choice in control of insect pests in Canadian forests (Addison, 1993), and it is used worldwide for control of agricultural pests (B.t. accounts for 90-95% of the total biopesticide market) (Feitelson *et al.*, 1992). Traditional B.t. spray-based products are relatively unstable in the environment but there are several new methods of delivery designed to overcome that problem (Feitelson *et al.*, 1992): toxin genes have been introduced into endophytic bacteria which colonize plant xylem, providing a type of systemic immunity; toxin genes have been encapsulated within *Pseudomonas fluorescens* cells, which are killed in fermentation after crystal formation (such bacteria cannot spread in the environment); toxin genes are incorporated into transgenic plants; plasmids containing B.t. genes have been transferred to other *B. thuringiensis* strains to broaden their host range (Feitelson *et al.*, 1992). Also, Bora *et al.* (1994) have transferred a toxin gene into a leaf-colonizing *Bacillus megaterium* strain and the genetically altered bacteria persist on leaves for up to 30 days.

## 1.9 Techniques used to study effects of B.t. δ-endotoxins on insect midgut

Recent research on efficacy and mode of action of toxins produced by strains of B.t. has relied on two tissue techniques, BBMVs and insect cell lines, while histological techniques provide pathological data at the cellular level and feeding bioassays are used to determine B.t. toxicity in insects. BBMVs are prepared by homogenizing midgut, then centrifuging the homogenate to retain only membrane fragments (Wolfersberger *et al.*, 1987). They are used primarily for midgut transport studies and toxin binding assays but results from binding studies do not always correlate with toxicity data in live insects (Gould *et al.*, 1992). Susceptibility of insect cells in culture has not always reflected that of midgut cells *in vivo* (Davidson, 1989). Apparent inconsistencies probably result from lack of suitable midgut epithelial cell lines, altered expression of receptors in cultured cells, and susceptibility of insect cells to other components such as solubilizing buffers. Whole tissue responses cannot be determined from examination of insect cells in culture (Davidson, 1989), and histological preparations of fixed tissue do not provide data on living tissue. Extensive use of feeding bioassays is expensive: they utilize many insects, and require time, space and appropriate diets (failure to develop artificial diets may prohibit laboratory rearings).

## 1.10 Objectives

The objective of this research is to develop an alternative method of determining effects of B.t. δ-endotoxins on midgut epithelial tissue. It involves development of a living tissue model which should provide a system more closely representative of conditions in the whole living insect than either cell culture or BBMVs. The procedure

developed here will produce a living, flat midgut epithelial tissue wholemount in which effects of B.t. δ-endotoxins can be examined directly in midgut epithelial cells. Production of this wholemount will provide a method to study effects of individual Cry toxins on all cells of the midgut epithelium and determine whole tissue response. It should also provide a quick and efficient means to screen various Lepidoptera for response to new toxins (new isolates or genetically engineered toxins), and to monitor insect populations for development of resistance to B.t. toxins.

Development of this technique will rely on the use of vital stains and fluorescence microscopy, which will allow direct observation on living cells as toxic effects occur. A description of cells and midgut tissue from untreated healthy insects will provide a basis for comparison after tissues are treated with B.t. toxins.

Although it is not an agricultural pest on the Canadian prairies, *Trichoplusia ni* (Hübner), the cabbage looper, was used in development of the model because it was the insect initially designated for use as the standard in all B.t. toxicity tests (Dulmage *et al.*, 1971) and it is susceptible to many B.t. δ-endotoxins.

Information presented here will include: a description of the procedure used to produce living epithelium tissue wholemounts; a description of tissue and midgut cells from *T. ni*; staining reactions of midgut cells using vital dyes before and after treatment with B.t. toxins; and comparison of effects of different individual cloned cry gene products on midgut cells.

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## Chapter 2 A new tissue technique for studying insect midgut epithelium

### 2.1 Introduction

The major route of entry for most insect pathogens is *per os* (through the mouth), so the site of action for many is the digestive tract. The cuticular lining in the fore and hindgut offers protection from many pathogens, but midgut, which is of endodermal origin, lacks this layer and is more susceptible to invasion by a variety of insect pathogens. Midgut is the site of digestion and nutrient absorption, and digestive enzymes and pH conditions often facilitate pathogen entry. Insect midgut is therefore the focus of research for many biological control strategies.

Concern over development of insect resistance and environmental impacts of synthetic insecticides has lead to increased research on biological control agents. One such agent is *Bacillus thuringiensis* (B.t.), a gram-positive soil bacterium with insecticidal properties. It produces insecticidal crystal proteins which bind to receptor proteins in midgut epithelial cells, insert into cell membranes to create pores, resulting in destruction of ion regulation and cell lysis (Knowles and Dow, 1993). Many strains of B.t. have been isolated which are active against Diptera, Coleoptera, and Lepidoptera. Safe effective methods of control for Lepidoptera are desirable since many are serious agricultural and forest pests. There are also several advantages in using Lepidoptera to study midgut response to B.t.: their digestive tract is uncomplicated (they lack digestive caecae so the digestive tract is a simple tube composed largely of one layer of epithelial cells resting on a basement membrane); there has been extensive research on their midgut physiology; and many are reared successfully in the laboratory. I am interested in using

B.t. to control insects such as bertha armyworm (*Mamestra configurata*) and diamondback moth (*Plutella xylostella*) which are lepidopteran pests of oilseed crops. Use of B.t. on both insects is problematic: *M. configurata* is a noctuid, many of which are weakly or not susceptible to B.t. (Martouret, 1961, cited in Heimpel, 1967; Morris, 1986), and *P. xylostella* has developed field resistance to many strains of B.t. (Tabashnik *et al.*, 1990; Whalon and McGaughey, 1993). *Trichoplusia ni*, the cabbage looper, is a noctuid which is highly susceptible to B.t. (Moar *et al.*, 1990; MacIntosh *et al.*, 1990). It will be used here for development of a midgut tissue assay which can then be adapted for use with bertha armyworm.

Techniques used to study effects of B.t. on Lepidoptera midgut include histological preparations of fixed midgut tissue, isolated midgut tissue, and brush border membrane vesicles (BBMVs). Results obtained from the latter two methods, using living tissue, are compared to toxicity assays using whole insects to determine their accuracy as experimental models.

Various authors (Harvey and Nedergaard, 1964; Wood and Moreton, 1978; Gupta *et al.*, 1985; Dow *et al.*, 1985; Harvey *et al.*, 1990) have adapted the Ussing chamber to study ion transport in lepidopteran midgut. In their apparatus, isolated midgut tissue is mounted in a chamber, bathed on both sides with physiological solutions, and stimulated with an electrical current to cause a 'short-circuit' which reduces the potential difference across the tissue to zero. Experimental parameters (bathing solutions, oxygen and nitrogen levels, pH, osmotic pressure) are all tightly controlled to maintain optimum tissue integrity. Harvey and Wolfersberger (1979) first reported use of a midgut chamber

to study effects of B.t. on isolated *Manduca sexta* midgut, and although optimum tissue vitality was maintained during their experiments, microscopical observations could not be made on all cells.

Most studies on mode of action of B.t. toxins do not use complete midgut but rely on cell membrane components (*e.g.* BBMVs) or cultured cells. Correlating toxicity data in whole insects with binding assays using cultured cells has proven difficult (*ibid.*, Knowles and Ellar, 1986), and probably is due partly to lack of suitable midgut epithelial cell lines (Davidson, 1989; Höfte and Whiteley, 1989). Johnson (1994) also demonstrated that membrane-binding in cultured insect cells does not always correlate with observed toxicity in those same cells. Baines *et al.* (1994) have recently established primary and continuous cultures of midgut epithelial cells from larval Lepidoptera although there are no published reports of their use in B.t. toxicity tests. Midgut-derived cell lines may be more appropriate for study, but whole tissue responses cannot be determined from examination of insect cells in culture (Davidson, 1989).

BBMVs are used as models in the study of toxin efficacy and binding characteristics (Gill *et al.*, 1992), and again, toxicity data in insects does not always agree with binding assay data using BBMVs. Wolfersberger (1990) found that although *Bacillus thuringiensis* subsp. *kurstaki* (B.t.k.) HD1-9 is almost 400 times more potent towards *Lymantria dispar* larvae than B.t.k. HD-73, the affinity of δ-endotoxin from HD-73 is much higher than that from strain HD1-9 in BBMVs. High-affinity binding correlated with low toxicity was also reported for activity of Cry1Ac against *Spodoptera frugiperda* (Garczynski *et al.*, 1991). It therefore seems likely that binding alone is not

sufficient for toxicity. Knowles and Ellar (1986) came to the same conclusion when they tested Cry1Ac binding to various cell lines: although Cry1Ac bound to many cell lines it killed only CF-1 cells. Johnson (1994) also demonstrated that membrane-binding in cultured insect cells doesn't always correlate with observed cellular toxicity. Another source of error may arise because comparisons are made between dissimilar larval instars. Many studies have correlated whole insect toxicity bioassays using first or third instars with BBMV binding assays from last instars (Van Rie *et al.*, 1989; Garczynski *et al.*, 1991; Gould *et al.*, 1992).

Use of BBMVs from *T. ni* has been limited: Estada and Ferré (1994) used them to study possible B.t. resistance mechanisms, and found that Cry1Aa did not compete with Cry1Ab or Cry1Ac for the same binding site in resistant *T. ni*.

A model which provides conditions more similar to tissue *in vivo* should provide more consistent and accurate results in toxicity tests than either cultured cells or BBMVs. However, development of such a model using whole midgut has been hampered by midgut architecture: the midgut is surrounded by layers of muscle, which when cut, cause the midgut to contract both longitudinally and dorso-ventrally, compressing the tissue into an inverted tube and rendering it useless.

Engelhard *et al.* (1991) describe production of flat epithelial wholemounts, in which basal lamina and connective tissue, including external gut muscles, are removed after the whole midgut is incubated in the proteolytic enzyme dispase. These investigators used double fluorescent labelling of fixed epithelial wholemounts to characterize columnar, goblet, differentiating, and stem cells in *T. ni*. Their technique

will be modified here to produce living epithelial tissue wholemounts, and vital dyes will be used to determine tissue viability.

## 2.2 Materials and methods

### 2.2.1 Insects

*Trichoplusia ni* larvae were maintained at 26°C ± 1°C under a 14L:10D photoperiod and fed on artificial diet containing antibiotics (0.5 g/l tetracycline, 0.5 g/l chloramphenicol) (Engelhard *et al.*, 1991). Second, third, fourth, and fifth instars, including moulting larvae, were selected for dissection (second and third instar larvae were used to identify cell types; third, fourth and fifth instar larvae were used to describe midgut folding patterns; all were used for tissue viability assays). Under these rearing conditions, insects moult approximately every two days. Larvae described as 'moulting' were in any stage of the process from cessation of feeding through ecdysis and consumption of cast exuvium. Once they resumed feeding they were classified as intermoult larvae. In some preparations, time of wholemount production after the moult was known, and in those cases, the consumption of cast exuviae signified the end of the moult.

### 2.2.2 Isolation of midgut epithelial tissue wholemounts

Dissections were performed in a sterile artificial hemolymph solution (SAHS) modified from *Calpodes ethlius* artificial hemolymph (Palli and Locke, 1987) by adding 48 µg/ml streptomycin sulfate, 30 µg/ml penicillin G sodium, 100 µg/ml ampicillin and 0.12 µg/ml amphotericin B, but omitting phenol red (Appendix 2). Wholemounts of midgut epithelial tissue were produced according to Engelhard *et al.* (1991), with slight

modification to maintain living epithelium for 24 h. Insects were placed dorsal side up, stretched slightly to maintain original length, and pinned through the head and the last abdominal segment. An incision was made beginning with a small nick at the posterior end and continuing anteriorly to the head capsule. Cut edges of the body wall were pinned open and the cavity was flooded with SAHS. Sterile surgical silk thread ligatures placed anterior to the cardiac valve and posterior to the pyloric valve isolated the entire midgut from the remaining alimentary canal. Cuticle under the alimentary canal was removed, leaving behind midgut secured by anterior and posterior pins. Cutting on the distal sides of the ligatures freed the midgut, which was removed, placed on a glass slide, oriented with the major, dorsal longitudinal muscle tract on top, and incubated in the proteolytic enzyme dispase (Grade II, Boehringer Mannheim Biochemicals, EC 3.4.24.4). (Slides were prepared previously by surrounding an enclosure with a hydrophobic perimeter and filling it with dispase. This enclosure remained intact throughout each experiment, containing a well of bathing medium.) Midgut was held in place by anchoring the ligature threads on surface-sterilized plasticine placed outside the fluid. Slides were labelled to distinguish anterior from posterior midgut. Midguts were submerged completely in dispase for 15 min, then incubated in fresh SAHS for 20 min (moulting insects required slightly longer incubation in the final SAHS bathing solution). Basal lamina and connective tissue, including associated fat body and tracheae, were teased away and discarded, revealing a faint demarcation where the major, dorsal longitudinal muscle tract was positioned (Figure 2.1). A longitudinal incision was made in the midgut along this line of demarcation. The cylindrical epithelium, now

unrestrained by muscle, unrolled, producing a flat tissue sheet. Peritrophic membrane and contents were removed, and the tissue rinsed again in SAHS. Time of day was recorded for the initial incision, addition of dispase, incubation period in SAHS, PM removal, treatment and observations. Tissues were treated with vital dyes or left untreated in SAHS. In preparation for these treatments, fluid was almost totally removed from bathing medium leaving only a minimal amount. Solutions then were added dropwise to the well without directly contacting the tissue (fluids were added or removed from tissue wells using sterile Tuberculin 1 cc syringes with 26G⅝ SubQ needles). Slides were rotated slightly to distribute added fluid and the time was recorded. Fluid levels were reduced just prior to examining tissues, and additional SAHS was added after examination was complete.

After treatment, tissues usually were oriented with their apical surface up. They did not attach to the glass substrate and remained floating in the bathing medium. Glass slides were placed in covered petri dishes containing moist filter paper and stored in a closed Plexiglas box containing sterile water to maintain humidity. Slides were maintained at ambient temperature (20 - 22°C).

### 2.2.3 Vital staining and tissue examination

Stains were prepared to final concentration by dilution with SAHS. Trypan blue (Sigma) was used either undiluted (0.4% in dipotassium phosphate and sodium chloride) or diluted to 0.01% or 0.02%. Nigrosin black (Matheson Coleman and Bell, C.I. 50420) was filter-sterilized (0.45 μm) and diluted to 0.2%.

Wholemounts used for cell identification and vital dye uptake were examined on a Reichert-Jung Polyvar microscope using brightfield microscopy with a variety of filters, or epifluorescent microscopy with an ultraviolet filter (U1). Tissues remained uncovered and floating in media. Removal of excess fluid from wells permitted use of 10x and 25x objectives, however not all areas of tissue were in focus at once. Wholemounts treated with vital dyes were examined along their entire length and observations on dye uptake into specific cell types was recorded for all regions of the midgut. Observations were made for up to 24 h post-treatment.

Tissue wholemounts used to describe midgut folding patterns and effects of dispase treatment were examined on a stereo microscope.

Colour micrographs were taken on Kodak Ektachrome Elite slide film and black and white micrographs were taken on Kodak TMAX. Slides and negatives were scanned into Adobe Photoshop using a Nikon Cool Scan. They were adjusted to reflect the original colour and contrast, and colour plates were printed on an Apple Color LaserWriter 12/600 PS.

### 2.2.4 Effects of dispase treatment

Effects of dispase treatment on midgut epithelial tissue from moulting larvae were determined by comparing trypan blue uptake into tissue from enzymatically treated and untreated midguts. Wholemounts from dispase-treated midguts were produced as described previously. Epithelium from untreated midguts was prepared as follows: midgut was removed after ligation of fore and hindguts, placed on dental wax, and opened with a lengthwise incision along the major, dorsal longitudinal muscle tract.

Minuten pins were placed through the cut edges to hold the tissue open and to minimize muscle contraction during dissection. Pinned midguts were submerged in SAHS in a covered petri dish. At 4 h post-dissection, tissues were flooded with 0.02% trypan blue for 5 min, then rinsed with SAHS. Observations of tissue staining with trypan blue from both enzymatically treated and untreated midguts were made for up to 24 h post-treatment with a stereo microscope.

## 2.3 Results

### 2.3.1 Description of midgut epithelial tissue

During observation, *T. ni* midgut epithelium was divided roughly into anterior, middle and posterior regions on the basis of cell type distribution and folding patterns. A central zone (cz), representing cells underlying the former, major, ventral longitudinal muscle tract, bisected the tissue longitudinally (Figure 2.2).

Folding patterns differed in each region of midgut and in each instar. Folding was absent in epithelium from third instar larvae and it increased slightly in newly-moulted fourth instar larvae (Figure 2.2). Sticky peritrophic membrane (PM) components (see below, and 2.4.1) pulled tissue into minor, irregular folds in the anterior region. Epithelium from fifth instar larvae had a complex folding pattern even after basal lamina and connective tissue removal. Deep folds in the posterior region formed villi (Figure 2.5), whereas folding was less structured in the middle (Figure 2.4) and anterior (Figure 2.3) regions.

Cell types easily identified in unstained epithelia were large, fully differentiated columnar epithelial cells (C), smaller putative differentiating cells (D), and goblet cells

(G) (Figures 2.6-2.8). Other cell types could not be distinguished without addition of vital stains. Columnar epithelial cells were dark when viewed through a daylight filter (DYLT) and transmitted light. A few (less than 10%), randomly situated, large, columnar cells extruded from the tissue with their numbers increasing slightly over time. These eventually lysed, releasing their contents into the media, and other cells appeared in their place.

Distribution of cell types produced patterns which differed from region to region and were more easily seen in epithelium lacking folds (taken from younger larvae). An area of demarcation was visible between posterior and middle regions of the epithelium from some second and early third instar larvae. This area, characterized by darker columnar cells in the posterior region, was not seen in epithelia from older larvae. Epithelia from the posterior region of second and third instar larvae contained columnar cells organized in a regular array (cells 10 - 15 μm apart), with both differentiating columnar cells and goblet cells filling in areas between (Figure 2.8). Numbers of large, fully differentiated columnar cells decreased from the posterior through middle regions (Figure 2.7) and in the anterior region (Figure 2.6) goblet cells dominated the epithelia. This pattern was obscured as cell numbers and folding increased in epithelia from older larvae.

Autofluorescence was observed in unstained epithelia when tissues were viewed through a UV filter. Colour in columnar cells, initially slightly green, faded rapidly on UV illumination. Columnar epithelial cells in the anterior and middle regions of some wholemounts had specks of bright blue autofluorescence in their cytoplasm (Figure 2.9).

This blue autofluorescence was seen in cells examined immediately and up to 9 h after wholemount production. Wholemounts prepared from intermoult third or fourth instar larvae had more fluorescing cells than those prepared from moulting larvae, but fluorescing cells were not present in all wholemounts. In some preparations this autofluorescence gradually diminished over time. Concurrent with this loss was the appearance of a layer of material over the tissue. This material initially appeared as an amorphous, sticky film overlying the tissue in the anterior and middle regions, often causing slight folds and ripples in the tissue if left undisturbed. With time it formed into a continuous sheet. An intact layer of material could be removed with forceps as early as one hour after wholemount incubation.

Two types of goblet cells were distinguished throughout the *T. ni* midgut epithelium. Goblet cells with basally-located cavities appeared stalk-like, each with a small clear droplet arising at the apical cell surface. Droplets discharged from the cells and sank to the bottom of the bathing fluid. Many droplets were observed in the bathing fluid shortly after tissue preparation, however they were also observed attached to goblet cells up to 9 h after wholemount preparation. Goblet cells with apically-located cavities appeared flattened in this preparation, with the cavity surrounded by a ring of cytoplasm. Discharging droplets were not observed from these cells.

Populations of cell types differed during the moult cycle of the insects dissected. Epithelial tissues from insects dissected during moulting had columnar epithelial cells of two types: large-sized fully differentiated cells and small basal cells. Insects dissected 12 to 13 h post-moult had medium- or large-sized columnar cells each ringed with smaller

undifferentiated cells. Differentiated cells increased in size until the end of the stadium, when large-sized columnar cells and small basal cells were present once again. During both moulting and the intermoult period some large columnar cells extruded apically above the tissue. These eventually lysed, releasing their contents into the media. Mature goblet cells were easily identified in tissues from moulting or intermoult larvae; differentiating goblet cells were indistinguishable from other differentiating cells.

### 2.3.2 Vital dye uptake

Trypan blue stained damaged cells along cut edges, some extruding columnar epithelial cells, and goblet cells within 10 min (Table 2.1). In the latter, staining was confined to their cavities and to droplets arising from their apical region. At high concentration (0.4%), trypan blue quickly stained many scattered, small, undifferentiated, basally situated cells in wholemounts prepared from insects dissected during moulting. At 0.02% staining occurred in the same cells but only after more than 3 h continuous exposure. Trypan blue was used at 0.01% or 0.02% in all subsequent experiments.

In epithelial wholemounts derived from insects dissected between moults, uptake of trypan blue occurred in damaged cells, goblet cell cavities, and extruding columnar epithelial cells. The remaining columnar epithelial cells remained unstained for up to 24 h. In goblet cells with basal cavities, apical droplets released into bathing media contained trypan blue, which remained sequestered in discharged droplets. When additional SAHS was added, blue stain was cleared from goblet cell cavities, and discharged stained droplets were replaced at the cell surface by colorless droplets. Goblet cells with apical cavities also removed stain, but droplet discharge was not

observed.

Addition of 0.2% nigrosin black to epithelial tissue from moulting larvae immediately stained some extruding columnar epithelial cells and droplets from goblet cells. Small basal cells appeared black within 15 min of nigrosin addition, and columnar epithelial cells began to darken within 30 min and were stained completely black within 24 h after wholemount production.

### 2.3.3 Effect of enzymatic removal of connective tissue

Tissues from untreated midguts were pinned on wax plates and viewed with incident lighting, which limited observations to the apical tissue surface only. Four hours after tissue preparation, untreated and enzymatically treated midguts were flooded with trypan blue. Staining patterns of columnar cells were similar in both preparations: a few extruding columnar epithelial cells in the middle and posterior regions were blue, as well as damaged cells along cut edges and at pin holes. Untreated midguts flooded with trypan blue and rinsed with SAHS at 24 h post dissection had approximately 25% blue cells (visual estimate), including extruding columnar epithelial cells and damaged cells.

Staining in goblet cells differed between treatments: there was no evidence of trypan blue staining in goblet cell cavities in untreated tissues, however cavities were stained transitorily in tissues prepared from enzymatically-treated midguts.

## 2.4 Discussion

### 2.4.1 Midgut epithelial tissue description

Microscopic examination of the apical surfaces of cells in epithelial wholemounts allowed identification of the predominant, fully differentiated cell types present in midgut

epithelium, columnar epithelial and goblet cells, as well as smaller differentiating cells. In living untreated and unstained wholemounts examined here, tightly packed columnar epithelial cells usually obscured more basally-occurring cells however numerous small, basal, presumably regenerative cells were seen in tissues dissected from insects during moulting and stained with concentrated trypan blue. These cells were not localized into nidi but were scattered throughout the epithelium, a pattern also reported for regenerative cells in *Hyalophora cecropia* midgut (Judy and Gilbert, 1970). Staining in these cells was observed before staining in fully differentiated columnar cells. Extruding columnar epithelial cells also stained with trypan blue before fully differentiated columnar cells. Their early staining indicated an alteration in membrane permeability, probably indicative of their degeneration. Baldwin and Hakim (1991) saw no evidence of cell degeneration or turnover of mature differentiated cells in *M. sexta* midgut, although cell degeneration and replacement of sloughed cells by differentiation of underlying regenerative cells normally occurs during each moult in insect midgut (Martoja and Ballan-Dufrançais, 1984). Here, some darkly-coloured columnar cells gradually enlarged (extruded) until they were sloughed off from the epithelium. I was unable to identify degeneration of any other cell types.

Goblet cells were identified by their spherical cavities. Staining was obvious in goblet cells after addition of either vital dye but it was transient, confined to the goblet cell cavity, and not observed after stained droplets were discharged unless additional dye was added to the bathing medium. Goblet cell nuclei remained unstained by trypan blue for up to 24 h after wholemount production.

In a previous midgut isolation technique, midgut stretching caused extrusion of goblet cell matrix plugs, resulting in decreased potassium transport over time *in vitro* (Schultz and Jungreis, 1977a; 1977b). In this technique, the midgut was anchored securely to maintain its original length during connective tissue and basal lamina removal, longitudinal dissection and peritrophic membrane removal. I observed release of small droplets from goblet cells even when the gut was not stretched. Droplets were stained by each vital dye added to the tissue (*e.g.* colour of attached droplets changed from clear to blue to clear over time after addition of trypan blue and rinsing with SAHS). Either every attached droplet was stained and cleared by each successive treatment, or successively produced droplets were coloured by different treatments. Since many discharged droplets were observed soon after wholemount production, and attached droplets were observed up to 9 h after tissue preparation it seems likely that goblet cells kept producing and discharging droplets.

Schultz *et al.* (1981) identified goblet matrix as an acid mucosubstance while Dow *et al.* (1984) suggest it is sulphated. Here, examination of droplets arising from goblet cells by scanning electron microscopy and energy dispersive X-ray analysis revealed no cellular structure, high amounts of potassium and chloride, and no sulphur (data not shown).

Blue autofluorescence was observed in large cells from some wholemounts. These cells were easily identified as columnar cells by switching back and forth from epifluorescence to transmitted light or by using both types of illumination simultaneously. Coincidental with some loss of blue autoflourescence from these cells

was the appearance of a sticky amorphous film which gradually formed a continuous layer above the midgut tissue. Since Adang and Spence (1981) describe peritrophic membrane (PM) synthesis in *T. ni* as the secretion of an amorphous material which matures into a fibrous matrix accompanied by aggregation and infiltration of amorphous material to the matrix, it seems likely that wholemounts were producing PM, and that the blue colour in columnar cells represented some PM component. Functional PM harvested from untreated *T.ni* fluoresces a faint blue (M. Erlandson, pers. comm.). In *T. ni*, the rate of PM production is the same as the rate of food passage (Adang and Spence, 1982), so perhaps nutrients in the bathing medium stimulated PM production in these wholemounts.

There were some features in tissues from younger larvae that were not evident in tissues from older larvae. The area of demarcation between the posterior and middle regions of the midgut in tissues from second and third instar larvae may represent the junction of anterior and posterior embryological midgut rudiments as described by the bipolar midgut rudiment theory (Mori, 1983). If this topography is correct, formation of the complete midgut occurs primarily from the anterior rudiment (approximately ⅔ vs ⅓ from the posterior rudiment). Columnar cell differentiation and proliferation obscured this line of demarcation as insects matured. Cioffi (1979) described a difference in goblet cell populations between the anterior-middle and the posterior regions of the midgut in *M. sexta*. This regionalization was not observed in *T. ni* although both types of goblet cells were present. She also noted structural changes in microvilli of columnar cells from the anterior to posterior regions, however at the resolution allowed here, no such

differences were noted.

Epithelial tissue folding patterns differed from region to region but were not similar to those seen in *M. sexta*, where midgut is composed of six corrugated strips separated from each other by an area of thin tissue, with a large longitudinal muscle running along each thin area (Cioffi, 1979). No such strips occur in *T. ni*, and longitudinal muscles are organized into two major tracts running along the dorsal and ventral surfaces of the midgut, and minor tracts located between major tracts. In *M. sexta* two series of folding in the anterior and posterior regions produce thicker epithelium than in the middle region, where there are only single folds (Cioffi, 1979). Here, folding was most pronounced in tissues from fifth instar larvae, decreasing in fourth instar larvae, and not present in third instar larvae. It was greatest in the posterior region and it decreased anteriorly. Because flat tissue surface was decreased due to presence of these deep folds in older larvae, earlier instar larvae were selected for use in wholemount preparation. Englehard *et al.* (1991) report progressive cell number increase from third to fifth instar, differences in regional cell populations in third and fourth instar, and a disproportionate increase in goblet cell population from third through fifth instar in fixed wholemounts from *T. ni*. Folds accommodated an increase in cell numbers in successive instars, and were maintained by connective tissue present between folds. Once connective tissue was removed by dispase treatment, larger folds remained due to pre-existing cell patterns and tissue structure.

### 2.4.2 Vital staining

Two dyes were tested here as indicators of tissue viability: nigrosin black and trypan blue. Nigrosin is an acid azine dye, used mainly as a dye for collagen in the textile industry (Baker, 1958). It has highest affinity for nuclear and granular cytoplasmic material, but more general staining occurs with exposure over time (Kaltenbach *et al.*, 1958). Kaltenbach *et al.* (1958) determined that stained cells are not necessarily dead, but have altered membrane permeability. Nigrosin is nontoxic to ascites tumor cells (0.2 to 0.5% in Ringer's medium) (Kaltenbach *et al.*, 1958), but here, 0.2% nigrosin in SAHS stained basal (regenerative) cells within 15 min and columnar cells within 30 min (2.3.2). These results were different from results obtained when tissues were stained with the traditional vital dye, trypan blue, and it appears likely that at the concentration tested here, nigrosin is toxic to midgut cells.

Trypan blue was used here as a marker for dead cells because it is the stain traditionally used in dead cell assays (0.1 - 0.2%). It does not stain most epithelial tissue (with the exception of kidney) (Evans and Schulemann, 1914), and it has been used to identify lepidopteran cells affected by B.t. δ-endotoxins (cell lines from *Choristoneura fumiferana*, *Spodoptera frugiperda* and *T. ni*) (Gringorten *et al.*, 1990; Thomas and Ellar, 1983; Gazit *et al.*, 1994). Living cells which take up benzidine dyes such as trypan blue are predominantly excretory or phagocytic cells (Evans and Schulemann, 1914) (Appendix 3). Results here showed trypan blue uptake was not confined to dead or damaged cells, but also occurred in goblet cell cavities, which were stained immediately on addition of dye. This was not a toxic reaction or the result of phagocytosis, but merely

evidence of the pumping activity in goblet cells. In early experiments, high concentrations of dye were toxic to basal cells, so less concentrated solutions were used in subsequent toxin assays.

### 2.4.3 Wholemount production

Epithelial tissue wholemounts produced by this technique were exposed to chemicals (bathing media, stain) not encountered *in vivo*. However, comparisons of tissue from enzymatically treated and untreated midguts showed no difference in trypan blue uptake into columnar cells, indicating that enzyme treatment did not damage these cells. There was no trypan blue seen in goblet cell cavities in tissues from untreated midguts whereas trypan blue routinely entered cavities in tissues from enzymatically-treated midguts. Perhaps basal lamina and connective tissue prevented basal access of trypan blue to goblet cells *i.e.* removal of basal lamina therefore allowed access of stains to goblet cell cavities in tissues treated with dispase. The $K^+$ pump located on the apical membrane of goblet cells pumps $K^+$ from the basal side of the cell into the goblet cell, through the cavity and out the valve to the gut lumen. Perhaps trypan blue is taken into the cell and transported into the cavity and out the valve in the same manner. Alternately, trypan blue also entered goblet cell cavities in untreated midguts, but more slowly, and it was not detected at the magnification used for viewing pinned midguts.

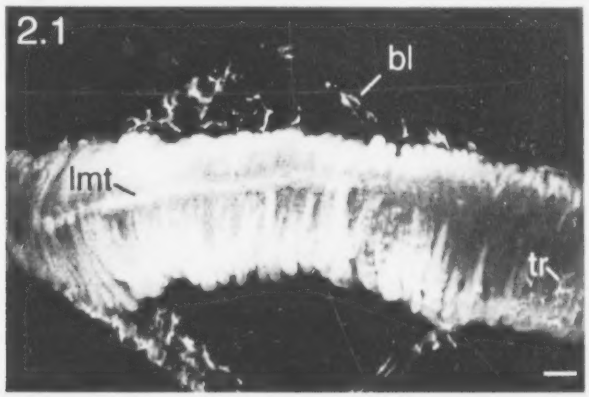
Epithelium prepared from enzymatically treated midguts remained alive for up to 24 h post-dissection as determined by exclusion of trypan blue, and continuous PM synthesis confirmed maintenance of some normal tissue function. Tissues from untreated midguts usually had more cell damage resulting from the pinning required to produce a

flat epithelial sheet.

## 2.5 Conclusions

In summary, I have described a technique to maintain midgut epithelium as a flat sheet for up to 24 h post-dissection in an artificial haemolymph solution. Use of such tissue wholemounts provides an efficient means to assay *T. ni* for toxic effects from any treatment that kills cells or alters their membrane permeability. Although tissue can remain viable for up to 24 h, assays should be completed as quickly as possible to minimize effects of cell deprivation. Tissue capacity would likely diminish over time since it is bathed only in artificial haemolymph which may lack important growth factors. Longer assay time would require additional nutritional input for optimum tissue viability. Addition of serum decreases diffusion of trypan blue (Pappenheimer, 1917) so it was not added to supplement SAHS in this system.

Figure 2.1 Removal of connective tissue and basal lamina from fifth instar *T. ni* larva after treatment with dispase. Connective tissue and basal lamina (bl), tracheae (tr), position of removed dorsal longitudinal muscle tract (lmt). Scale bar = 500 μm.



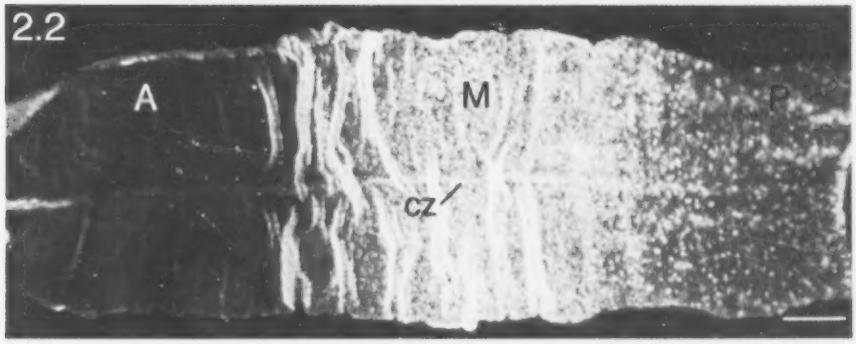




Figure 2.2 Epithelial tissue wholemount from newly-moulted fourth instar *T. ni*. Note anterior (A), middle (M) and posterior (P) regions, central zone (cz) representing position of removed dorsal longitudinal muscle tract. Scale bar = 500 μm.

Figures 2.3 - 2.5 Midgut epithelium wholemount from newly-moulted fifth instar *T. ni* larva. Scale bar for all three micrographs = 750 µm.

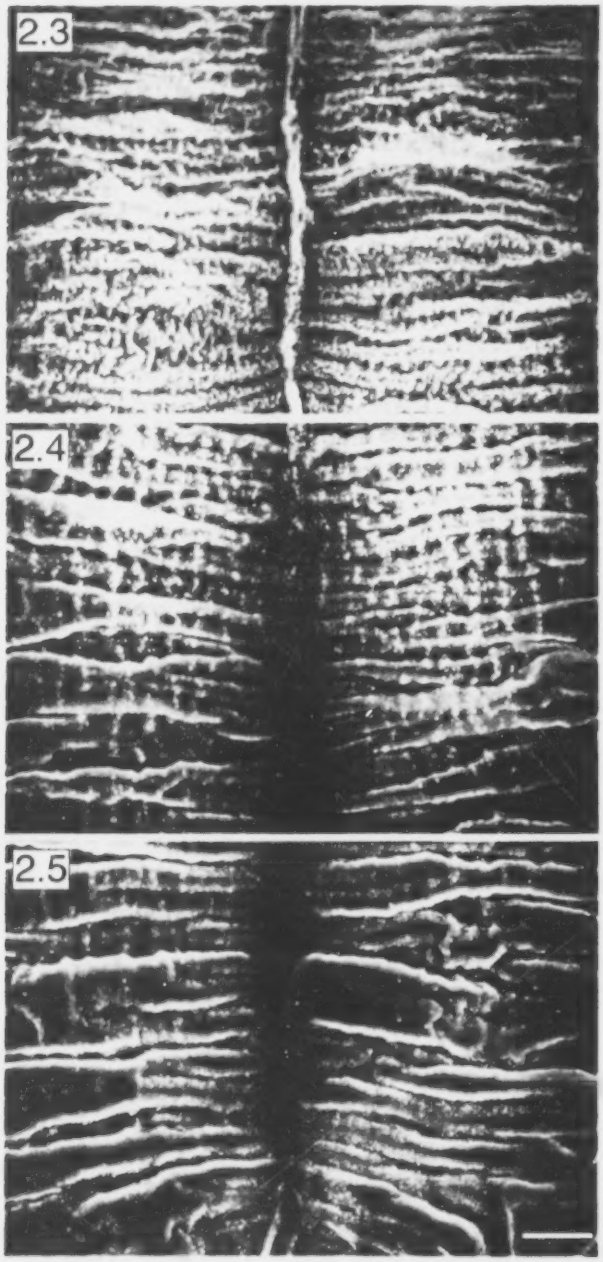



Figure 2.3 Anterior region
Figure 2.4 Middle region
Figure 2.5 Posterior region. Note deep folds.

Figures 2.6 - 2.8 Topological view of unstained cells from *T. ni* second instar larva. Note goblet cell (G), columnar epithelial cell (C) and differentiating cell (D) (DYLT filter). Scale bar for all three micrographs = 50 µm.

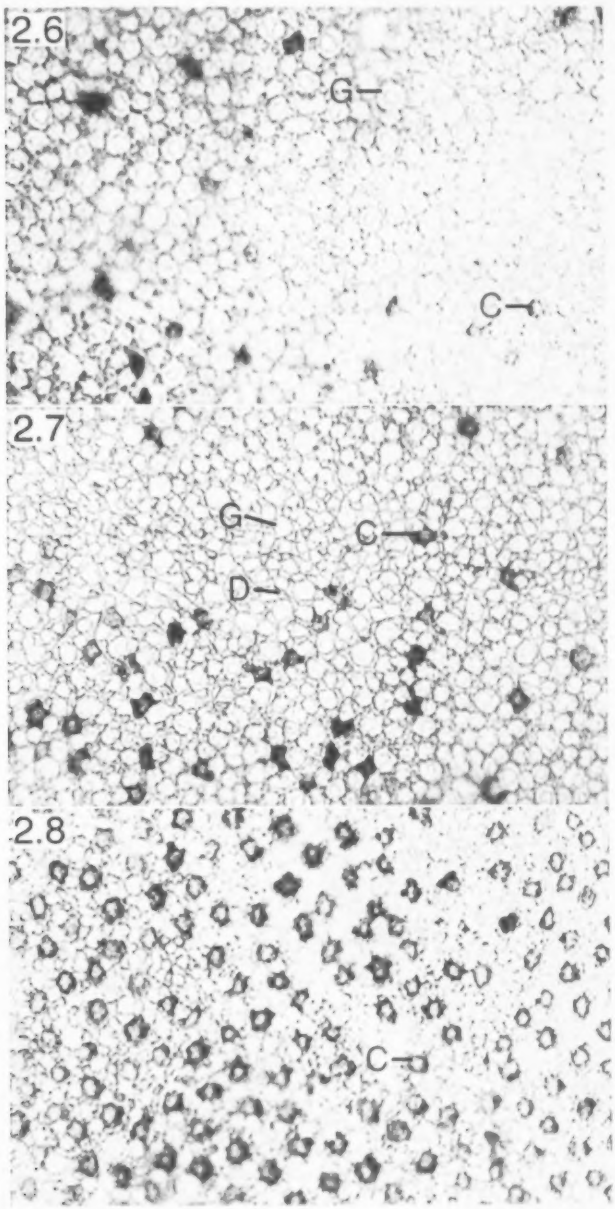



Figure 2.6 Anterior region.
Figure 2.7 Middle region
Figure 2.8 Posterior region

Figure 2.9 Blue autofluorescence present in unstained columnar epithelial cells from middle region of intermoult fourth instar *T. ni* midgut. Note columnar epithelial cell (C) and goblet cell (G) (U1 filter).
Scale bar = 25 μm.

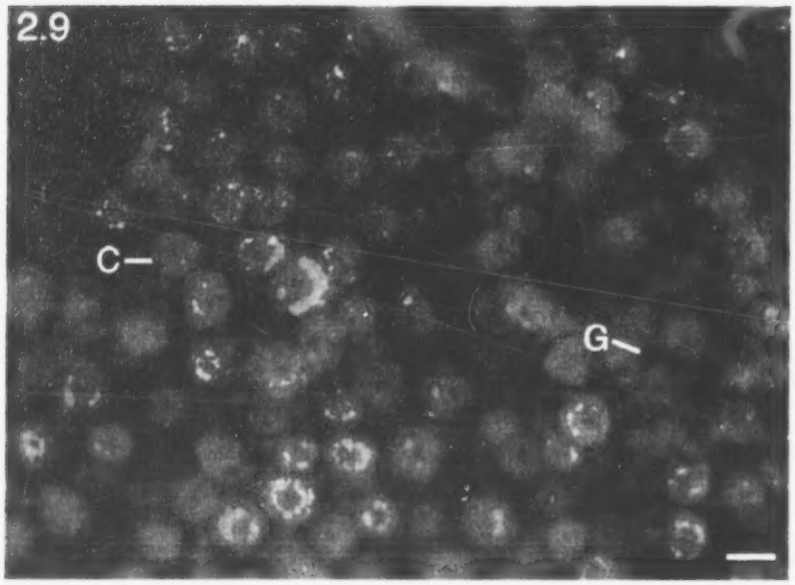


Table 2.1. Vital dye uptake into midgut epithelium cells from moulting or intermoult *T. ni* larvae.

| | Moult | | | | | Intermoult | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| STAIN | edg | col | ext | gob | bas | edg | col | ext | gob | bas |
| Trypan blue 0.40% | + | - | ± | [+] | + | + | - | ± | [+] | |
| Trypan blue 0.02% | + | - | ± | [+] | +[1] | + | - | ± | [+] | |
| Trypan blue 0.01% | + | - | ± | [+] | - | + | - | ± | [+] | |
| Nigrosin black 0.20% | + | + | ± | [+] | + | | | | | |

| | |
|---|---|
| edg | dead or damaged cells along cut edges |
| col | columnar epithelial cells |
| ext | extruding columnar epithelial cells |
| gob | goblet cells |
| bas | basal cells - presumably regenerative |
| + | stain uptake into cell |
| - | no stain uptake into cell |
| ± | stain uptake into some cells |
| [+] | stain uptake confined to goblet cavity; transitory |
| [1] | stained after > 3 h exposure |

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## Chapter 3 Effects of *Bacillus thuringiensis* Cry1Ac on larvae and midgut epithelial wholemounts produced from *Trichoplusia ni*

### 3.1 Introduction

*Trichoplusia ni*, the cabbage looper, was designated as the standard insect for all *Bacillus thuringiensis* (B.t.) toxicity tests because it was found to represent the response of broad groups of insect species to B.t. (Dulmage *et al.*, 1971). Its use has been continued in bioassays using new B.t. isolates or single gene products, however its use in model systems (*e.g.* brush border membrane vesicles and cell cultures) to study binding and mode of action is limited.

Toxicity of B.t. single gene products, and specifically Cry1Ac, in *T. ni* has been determined through feeding bioassays. Moar *et al.* (1990) found that $LC_{50}$s[1] for neonate *T. ni* fed *ad libitum* with diet-incorporated *Bacillus thuringiensis* var. *kurstaki* (B.t.k.) Cry1Ac protoxin varied depending on the strain from which Cry1Ac was derived. They obtained $LC_{50}$s of 0.10, 0.12, and 0.31 µg/ml diet when mortality is scored at day seven for Cry1Ac derived from B.t.k. strains NRD-12, HD-1, and HD-73 respectively. MacIntosh *et al.* (1990) also used diet-incorporated B.t.k. HD-73 containing the single insecticidal protein Cry1Ac and found the $LC_{50}$ to be 0.09 µg/ml when mortality is scored at day 6. In a diet surface contamination assay of trypsin-activated Cry1Ac in neonate *T. ni*, the $LD_{50}$ was 0.32 µg/cm$^2$ diet (Estada and Ferré,

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[1] $LC_{50}$ (lethal concentration) is the concentration of substance in a medium necessary to kill 50% of the population; usually expressed as parts per million of medium or per individual. $LD_{50}$ (median lethal dose) is the dose necessary to kill 50% of the population to which it is administered; usually expressed as amount per individual or per unit body weight. $ED_{50}$ (median effective dose) is the dose which will produce a response in half the population (Faust, 1974; Pedigo, 1989).

1994). Ge *et al.* (1989, 1991) supplied measured amounts of Cry1Ac crystals in diet surface contamination assays to neonate, second or third instar larvae and determined $LD_{50}$s of 0.32 μg for second and third instar larvae scored at day 9 and 0.43 μg for neonates scored at day 5 post-treatment.

Murphy *et al.* (1976) first demonstrated the toxic activity of B.t. δ-endotoxins from B.t.k. HD-1 on cultured insect cells, including TN-368, a cell line derived from adult *T. ni* ovaries. Within 15 min of treatment affected cells swelled, cell membranes thickened and cytoplasmic refractility changed. Cytotoxic activity was maximum within 1 h as cells became vacuolated and many lysed. TN-368 cells have been used also to study mode of action and ion transport (Himeno *et al.*, 1985) and cytopathologic effects (Nishiitsutsuji-Uwo *et al.*, 1979; McCarthy, 1994) of B.t. δ-endotoxins. Nishiitsutsuji-Uwo *et al.* (1979) found swelling of TN-368 cells to be dependent to some extent on the ionic composition of the solution used to dilute the toxin. They also demonstrated that by replacing medium containing activated toxin with fresh uncontaminated medium, swollen cells were able to recover, from which they concluded that effects of δ-endotoxin are reversible if caught in time.

Estada and Ferré (1994) used brush border membrane vesicles (BBMVs) from *T. ni* to study possible resistance mechanisms. They correlated toxicity of B.t. insecticidal crystal proteins (ICPs) in neonate *T. ni* larvae with binding to BBMVs and demonstrated that a line of *T. ni* selected for Cry1Ab developed resistance to Cry1Ab but not the closely related Cry1Aa or Cry1Ac.

Although both cell cultures and BBMVs provide model systems which simulate

conditions *in vivo*, neither provides a complete physiological mimic of midgut epithelium (in fact most cell lines are not even derived from midgut cells). A new model system has recently been described (Chapter 2) in which living, isolated epithelial wholemounts are produced from *T. ni* midgut. These have been used here to identify cells affected by B.t. toxins. Solubilized Cry1Ac, which is highly toxic to *T. ni* (Moar *et al.*, 1990; MacIntosh *et al.*, 1990), was applied directly to the medium bathing epithelial tissues and its effects were monitored microscopically over time using the vital stains trypan blue, propidium iodide (PI), acridine orange (AO), and 4',6-diamidino-2-phenylindole (DAPI). Cells affected by B.t. were identified by their incorporation of trypan blue, PI and DAPI. Three types of insect bioassays were performed to confirm toxicity of Cry1Ac to *T. ni*: a diet surface contamination feeding bioassay, an oral inoculation bioassay, and wholemount production following an inoculation bioassay. This living midgut epithelium tissue provides a rapid and sensitive assay to determine insect response to B.t. toxins, and a new model for additional studies on mode of action of B.t. toxins in Lepidoptera.

## 3.2 Materials and methods

### 3.2.1 Insects

*Trichoplusia ni* larvae were maintained as described previously (2.2.1). Third and fourth instars, including moulting larvae, were selected for dissection. Insects were assigned to time zero after moulting when they started to consume cast exuviae.

### 3.2.2 Bacteria culturing and protoxin purification

Cultures of *Escherichia coli* containing the NRD-12 Cry1Ac expression plasmid were kindly provided by L. Masson, Biotechnology Research Institute, National Research Council. Cry1Ac inclusion bodies were produced and purified according to Masson *et al.* (1989) (Appendix 4.1). Purified inclusion bodies were solubilized in 50 mM sodium carbonate/HCl pH 9.5 and 10 mM dithiothreitol (DTT) for 60 min at 37°C (Huber *et al.*, 1981). Insoluble material was removed by centrifugation at 10,000 *g* for 5 min. Protoxin production was verified by SDS-PAGE and protein concentration was determined by the Bradford assay using BSA as a standard.

*E. coli* strain HB101 was grown in LB broth (Ausubel *et al.*, 1992) and cultures were processed as above with the following exception: a dense layer of cell components remained on top after centrifugation using a low-density Renografin gradient. There was no pellet formed. This top layer was retained and washed with sterile water through two centrifugations at 11,000 rpm for 40 min at 4°C.

### 3.2.3 Insect bioassays

#### 3.2.3.1 Feeding bioassay

Individual larvae were isolated during the moult to third instar and deprived of food for 13 to 16 h. Each insect was then placed in a plastic cup with a 5 × 5 mm disk of diet, either untreated or inoculated with 10 µl of purified Cry1Ac inclusion bodies. Twenty-five larvae were tested at each of seven concentrations ranging from $6.33x10^{-1}$ to $6.33x10^{-7}$µg of protein per insect. Each insect was transferred to a new diet cup after consuming the diet. Insects were incubated in the dark at 27°C ± 2°C

and monitored daily for 18 days or until adult emergence. Necropsies were performed on each dead insect to identify pathogens present. Data were analyzed using probit analysis (Finney, 1971) after correction for control mortality with Abbott's formula (Abbott, 1925).

#### 3.2.3.2 Oral inoculation bioassay

Larvae were selected during the moult to third instar and divided into three groups after moulting. Group I larvae were isolated immediately to prevent each from feeding on its cast exuvium, group II larvae were allowed to eat cast exuviae only, and group III larvae were allowed to eat cast exuviae and were supplied with artificial diet. Larvae from groups I and II were isolated individually in plastic cups to prevent cannibalism while 10 to 15 larvae from group III were kept per cup with artificial diet provided *ad libitum*. All larvae were inoculated at the same time post-moult: those from groups I and II being kept without food for 17 h while those from group III were fed for 15 h, then isolated as individuals and kept without food for 2 h before inoculation.

Larvae were inoculated *per os* (Keddie and Volkman, 1985) with 2 µl of Cry1Ac protoxin containing 0.4 µg of protein. Control larvae were inoculated with 2 µl of sterile artificial hemolymph solution (SAHS) (Appendix 2). Larvae dying within 24 h of inoculation were discarded (less than 5%). Larval weights were recorded before inoculation and at 24 h intervals until pupation (moulting larvae were not weighed); final pupal weight was recorded 24 h after the pupal moult. Insects were monitored daily until eclosion or death. Mortality data was corrected using Abbott's formula

(Abbott, 1925).

### 3.2.3.3 Oral inoculation followed by midgut assay

After the moult to fourth instar, individual larvae were placed either on artificial diet or deprived of food (*i.e.* not allowed to eat exuvium or diet). Insects were inoculated *per os* with 5 µl of Cry1Ac protoxin (containing 0.95 µg protein) or 5 µl of solubilization buffer. After inoculation, all insects were fed artificial diet. Midgut epithelial wholemounts were produced from inoculated larvae and stained with DAPI at various times post-inoculation.

### 3.2.4 Isolation of midgut epithelial tissue

Midgut epithelial wholemounts were produced as described previously (2.2.2). It was possible to orient tissues with either their apical or basal surface up, but since staining results in all treatments were independent of tissue orientation, tissues were usually oriented apical surface up. Slides were placed in covered petri dishes containing moist filter paper and stored in a closed Plexiglas box at ambient temperature (20 - 22°C) in the dark (slides were kept in the dark to protect fluorochrome-treated tissues).

### 3.2.4.1 Vital staining

All stains were prepared to final concentration by dilution with SAHS. Trypan blue (Sigma) was diluted to 0.01% or 0.02%. (It was also used undiluted in some wholemounts to purposefully stain basal cells). Acridine orange (AO) (Molecular Probes) and propidium iodide (PI) (Sigma) were prepared from 1 mM, sterile-filtered, aqueous stock solutions. AO uptake was assessed at concentrations of 2.5, 10, 25, 50,

and 100 μM and PI at 10, 25, 50, and 75 μM. DAPI (Molecular Probes) 1 mM aqueous stock was diluted to 1 μM for use. Published staining reactions of these vital dyes in living, dead or dying cells from various sources are provided in Table 3.1.

Vital stains were added singly and in combination to midgut epithelial wholemounts at various times after final rinse in SAHS. In most cases stains remained in the bathing media for the duration of the observation period. Tissues were examined within 24 h post-treatment on a Reichert-Jung Polyvar microscope equipped for brightfield, differential interference-contrast, and epifluorescent microscopy. Trypan blue uptake was observed using brightfield microscopy; that of AO, PI, and DAPI with epiillumination and a U1 ultra-violet (BP 330-380) filter. Some observations of PI uptake were done using a G2 (BP 520-560) filter. Observations made using UV illumination were performed in the dark and were limited to less than 5 min each. Simultaneous exposure to epifluorescence and brightfield illumination with a variety of filters was used when taking some micrographs. Slides were scanned into Adobe Photoshop using a Nikon Cool Scan. They were adjusted to reflect the original colour and contrast, and colour plates were printed on an Apple Color LaserWriter 12/600 PS.

#### 3.2.4.2 Control treatments on isolated midgut epithelial tissue

Differences in cell permeability to vital stains due to exposure to UV radiation, age of wholemount tissue post-dissection, solubilization buffers and *E. coli* HB101 cell components were assessed by staining reactions at various times post-treatment.

Vital stains were added singly and in combination to midgut epithelial

wholemounts at various times after final rinse in SAHS and tissues were examined within 24 h post-treatment. Aliquots of *E. coli* cell components were diluted with sterile water and applied simultaneously with DAPI to midgut epithelial tissue, and observations were made up to 5 h post-treatment. Toxin solubilization buffer was applied to midgut epithelial tissue with either 10 µM PI or DAPI.

#### 3.2.4.3 Toxin treatments on isolated midgut epithelial tissue

Inclusion bodies or protoxin stock solutions (0.63 and 0.20 µg/µl protein respectively) were diluted in SAHS and applied (1 to 4 µl) to midgut epithelial tissue wholemounts at various times after the final rinse in SAHS. Time of treatment (post-moult) was recorded for each tissue sample.

Stains were added singly or in combination to midgut epithelial tissue at various times after final rinse in SAHS, either before, with, or after treatment with inclusion bodies or protoxin. Midgut epithelial cells were examined at various times for up to 24 h post-treatment.

## 3.3 Results

### 3.3.1 Insect bioassays

#### 3.3.1.1 Feeding bioassay

Insect bioassays were performed to verify that Cry1Ac produced here was active in *T. ni*. The concentrations tested were not replicated. For Cry1Ac inclusion bodies, the $LD_{50}$ for *T. ni* was 0.10 µg if larvae were scored for mortality at day seven and 0.01 µg when scored after adult emergence. A *Micrococcus* sp. was recovered from necropsies performed on inoculated insects (recovery of *Micrococcus* from inoculation

rates of $6.33\times10^{-1}$ and $1.27\times10^{-1}$ µg inclusion bodies was 100% and 60% respectively). Subsequent examination of the *T. ni* source culture confirmed presence of these bacteria in larval midguts, and the artificial diet was modified to include antibiotics to control this.

#### 3.3.1.2 Oral inoculation bioassay

Mortality data for third instars inoculated with SAHS or 0.40 µg Cry1Ac protoxin are shown in Table 3.2. Although numbers in each treatment were low, slight toxic effects of Cry1Ac protoxin were demonstrated in group III. Mortality for third instars inoculated with 0.40 µg Cry1Ac protoxin was highest in group III (51%, corrected for control mortality), and was significantly different from that of insects inoculated with SAHS (P = 0.01, Chi-square test). There were no significant differences among treatments for larval weight 5 d post-inoculation, pupal weight, days to pupation or days to eclosion (P = 0.05; Student's *t*-Test).

#### 3.3.1.3 Oral inoculation followed by midgut assay

Fed insects inoculated with solubilization buffer and dissected for production of midgut epithelial wholemounts at about 4 h post-inoculation contained DAPI in a few, large, extruding columnar epithelial cells and in damaged cells during the period of observation (6 to 20 h post-inoculation) (Table 3.3). In wholemounts produced from insects dissected at 27 h post-inoculation, DAPI was observed in damaged cells only, with transient appearance in goblet cells.

In fed insects inoculated with Cry1Ac protoxin and dissected 3 to 4 h post-inoculation, DAPI was present in columnar epithelial cells in the anterior two-thirds of

the midgut and in damaged cells. Insects inoculated more than 24 h after the moult and dissected 6 h post-inoculation displayed DAPI staining only in damaged cells and some extruding columnar epithelial cells, with transient appearance in goblet cells. In insects dissected at 28 h post-inoculation, DAPI was limited to some extruding columnar epithelial cells and damaged cells.

Epithelial wholemounts could not be prepared 3 h post-inoculation from insects unfed before receiving protoxin, although they could be prepared routinely from unfed control insects. By 27 h post-inoculation, insects inoculated with protoxin had eaten artificial diet, and wholemounts were prepared easily. DAPI was observed in damaged cells only.

Eighty per cent of all inoculated insects were alive 4 d after inoculation. Those receiving protoxin, however, showed feeding inhibition the first 24 h post-inoculation (data not shown).

### 3.3.2 Control treatments on isolated epithelial tissue

#### 3.3.2.1 Vital dye uptake

Colour reactions of vital stains in healthy or damaged columnar epithelial cells from midgut tissues treated with SAHS are compiled in Table 3.4; uptake of vital dyes into individual cell types from wholemounts treated with SAHS is shown in Table 3.5. Trypan blue uptake was reported previously (2.3.2): healthy cells remained unstained whereas damaged cells along cut edges and some extruding columnar epithelial cells stained blue. Trypan blue staining was transitory in goblet cell cavities.

Addition of 2.5 μM AO stained all extruding columnar cells green but staining

was minimal in other cell types. Droplets arising from goblet cells were either green or red, although no red colour was detected in any cells. AO (10 μM) stained both nuclei and cytoplasm of many cells green, with red flecks present in columnar cell cytoplasm and in goblet cell cavities. Goblet cells appeared dark grey, although droplets arising from stalk-like goblet cells were green. When 50 or 100 μM AO was used, some extruding columnar epithelial cells were orange, as were cells along cut edges. Goblet cells had green cytoplasm with yellow or dark red cavities. Stain in goblet cell cavities created a bright red lattice pattern; droplets arising from goblet cells were red, orange or green.

When 10, 25, or 75 μM PI was added to epithelial tissues, damaged cells along cut edges became red. Goblet cell cavities stained red, creating a basal lattice pattern. When the tissue was washed gently with SAHS, red droplets were released to the media, and by 45 min post-staining, basal staining was gone. Most columnar cells remained unstained.

Although nuclei of nearly all epithelial cells treated with DAPI remained unstained even after 24 h exposure, cell cytoplasm gradually became faint blue. Damaged cells and a few extruding columnar epithelial cells had blue nuclei (Figure 3.1; arrow). This was distinct from the blue autofluorescence seen in the cytoplasm of some columnar epithelial cells from the anterior and middle regions of the midgut (2.3.1, Figure 2.9). Goblet cell cytoplasm remained unstained while numerous goblet cell cavities appeared faint blue. Droplets released from goblet cell cavities were pale blue in U1 and clear in DYLT.

### 3.3.2.2 Stain combinations

When both trypan blue and AO were applied to midgut epithelial tissue from intermoult larvae treated with SAHS alone, large extruding columnar epithelial cells were blue in DYLT and green with small yellow and orange granules in their extruded contents in U1. Damaged cells in these tissues stained blue after addition of trypan blue and red after addition of PI. Basal cells stained blue by exposure to concentrated trypan blue became purple or red after addition of PI and AO. (Both trypan blue and PI are used to identify damaged or dead cells, *i.e.* cells with altered membrane permeability.)

After simultaneous application of AO and PI, healthy columnar epithelial cells appeared green and damaged cells were red (Table 3.4) (Figure 3.3). Occasionally some columnar cells on ridges or in folds were rusty to red. Goblet cell cavities and droplets arising from goblet cells were red or green. When PI was applied first, goblet cell cavities were red; after the addition of AO these cavities were green.

Addition of vital dyes revealed subtle cell patterns not observed previously. In a wholemount prepared from a newly moulted fourth instar *T. ni* larva and stained with AO and PI small basal cells appeared in a nest-like configuration (Figure 3.4). Nests were observed scattered throughout the posterior region; each had a central core which lacked fully differentiated columnar cells.

### 3.3.2.3 Application of solubilization buffer

There was no difference in PI uptake between epithelial tissue from just-moulted larvae treated with protoxin solubilization buffer (sodium carbonate-HCl/DTT) and

tissue treated with PI alone. Goblet cell cavities appeared slightly pink. Cells in epithelial tissue treated with solubilization buffer remained unstained by DAPI up to 8 h post-treatment except damaged cells along cut edges, which had blue nuclei.

#### 3.3.2.4 *E. coli* HB101 culture products

Application of *E. coli* HB101 cell components to midgut epithelial tissue in SAHS did not increase DAPI staining: only some extruding columnar epithelial cells and damaged cells along cut edges were stained.

### 3.3.3 Toxin treatments on isolated midgut epithelial tissue

#### 3.3.3.1 Non-fluorescent vital dye uptake

When trypan blue was added with Cry1Ac protoxin to epithelial tissue from moulting larvae, small scattered basal cells appeared blue within 3.5 h (Table 3.5) and although columnar cells were enlarged, no stain was detected at that time. By 24 h post-treatment both basal and columnar epithelial cells stained blue. Addition of more than 1 μg protoxin caused tissue disintegration within 8 h.

#### 3.3.3.2 Fluorescent vital dye uptake

Uptake of fluorescent vital dyes into cells after treatment with Cry1Ac protoxin is shown in Table 3.5. When epithelial tissues prepared from intermoult larvae were treated with PI followed by Cry1Ac protoxin, cytoplasm of some columnar epithelial cells, especially extruding cells, became rusty coloured within 10 min. The number of stained columnar cells increased until, by 24 h post-treatment, almost all were red. With tissues prepared from moulting larvae, a regular array of rusty coloured basal cells appeared within 30 min after this treatment. Within 2½ h all fully differentiated

columnar epithelial cells were red also. In many wholemounts columnar epithelial cells in anterior and middle regions retained flecks of blue autofluorescence.

There were more extruding columnar cells in tissues treated with Cry1Ac than in tissues treated with SAHS alone. Fully differentiated columnar cells became visibly swollen and with time many detached from the tissue.

No immediate colour change was observed in columnar epithelial cells previously stained green by AO after addition of Cry1Ac protoxin. Goblet cell cavities changed from green to orange. Over time columnar epithelial cells became pale yellow.

Nuclei in columnar epithelial cells fluoresced blue within 2 h of DAPI addition following treatment with Cry1Ac protoxin. Simultaneous use of U1 and DYLT filters showed that tissues taken from intermoult insects and treated with Cry1Ac protoxin plus DAPI had blue nuclei predominantly in medium sized cells, with only a few small cells stained (Figure 3.2). Tissues from larvae dissected during moulting had DAPI in nuclei from both large columnar epithelial cells and small scattered basal cells. This staining often produced a circular pattern in which large fully differentiated columnar cells were ringed with smaller basal cells (Figure 3.5). Goblet cells were enlarged with faint blue cavities and unstained nuclei. (Differences in midgut cell populations between moulting and intermoult larvae are described in 2.3.1.)

Blue nuclei occurred in most columnar epithelial cells when observed 3 h after DAPI had been added to tissues treated with Cry1Ac 15 to 27 h earlier.

#### 3.3.3.3 Stain combinations

Simultaneous addition of PI and AO with Cry1Ac protoxin to midgut epithelial tissues resulted in rusty-coloured fully differentiated columnar epithelial cells on a green background within 2 h (Figures 3.6 and 3.7). All columnar epithelial cells in tissues stained with PI and DAPI showed red or blue nuclei within 20 min after the addition of Cry1Ac protoxin. Orange, yellow, or pink cells, interspersed with green goblet cell cavities were observed after subsequent addition of 10 μM AO.

#### 3.3.3.4 Addition of Cry1Ac inclusion bodies

Addition of insoluble Cry1Ac inclusions to one epithelial tissue wholemount resulted in DAPI staining of nuclei in columnar epithelial cells within 2 h. Goblet cells were visibly enlarged, but their nuclei remained unstained.

#### 3.3.3.5 Peritrophic membrane and basal lamina synthesis

Newly formed PM occasionally prevented access of toxin, stain, or both to columnar epithelial cells. Peritrophic membrane production occurred 30 to 60 min after tissue preparation in epithelial tissues taken from moulting larvae (its production coincident with disappearance of some blue autofluorescing flecks from columnar cells) (2.3.1). Manual removal of newly synthesized PM from anterior and middle regions resulted in DAPI uptake within 1 h into previously unstained columnar epithelial cells treated with Cry1Ac. Uptake into columnar cell nuclei from the posterior region had already occurred. Columnar cells in that region did not exhibit blue autofluorescence.

On some preparations, basal lamina was observed after toxin treatment induced

release of epithelial cells (Figure 3.8, 24 h post-treatment). Tissue preparations made shortly after dispase treatment and examined by scanning electron microscopy lacked basal lamina (pers. comm., B. A. Keddie).

#### 3.3.3.6 UV radiation effects on vital dye uptake

Observations of fluorescent dye uptake into cells affected by Cry1Ac protoxin were made immediately and at 2.5 h after dye addition on some preparations, and at 2.5 h only after dye addition in others. No differences were seen in dye uptake patterns, indicating that initial exposure to UV radiation did not cause increased dye uptake. Untreated tissue showed no difference in DAPI incorporation during multiple observations taken over 24 h.

## 3.4 Discussion

### 3.4.1 Vital staining

Trypan blue was used initially in development of this tissue model and was acceptable to show nonviable cells in untreated tissue (also 2.3.2). There were problems using it as a dead-cell indicator after addition of Cry1Ac however: it was difficult to observe blue dye uptake into dark columnar epithelial cells until they were heavily stained after many hours exposure; results in basal cell staining were inconclusive since trypan blue appeared toxic to those cells after several hours exposure (2.3.2); and once many columnar cells were stained, staining in goblet cells merely confused interpretation. Therefore the fluorescent dyes AO, PI, and DAPI were evaluated as indicators of cell viability after addition of B.t. toxin.

Acridine orange is a pH sensitive metachromatic dye that stains living cells green

(Bank, 1987) and dying cells yellow-orange (West, 1969). Propidium iodide stains dead or damaged cells red and does not penetrate healthy, unfixed cells (Crissman *et al.*, 1979; Bank, 1987; Arndt-Jovin and Jovin, 1989). Bank (1987) used AO and PI together to differentiate between intact and compromised plasma membranes in pancreatic islet cells. He compared staining with trypan blue and AO/PI and found that the AO/PI assay was stable, reproducible, accurate, and non-toxic while the trypan blue assay was not (the percentage of cells staining with trypan blue increased with time, limiting the window of staining to 5-10 min). Although the AO/PI combination worked well for midgut tissue preparations bathed in SAHS alone, AO staining sometimes obscured PI uptake into cells affected by Cry1Ac, making assessment of toxic effects difficult when low concentrations of toxin were used.

DAPI is used routinely after cell fixation to show DNA content in nuclei (Arndt-Jovin and Jovin, 1989). It is normally excluded from unfixed, healthy cells (Taylor and Milthorpe, 1980; Tanke *et al.*, 1982) except for Chinese hamster ovary cells (Lydon *et al.*, 1980). With this technique, DAPI stained only dead and damaged cells in tissues treated with SAHS alone. It was chosen for future research on effects of B.t. toxins for several reasons: it was excluded from healthy (*i.e.* untreated) cells; it entered columnar epithelial cells after treatment with Cry1Ac; and uptake into stained nuclei of affected cells was observed easily[2].

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[2] Staining results were similar for both DAPI and PI but for very fast results PI would be preferred. Tissues would then be observed using the G2 filter (BP 520-560) because PI staining was visible within 5 min of treatment with Cry1Ac. DAPI typically required a longer time to accumulate to visible concentration under UV illumination. The G2 filter was not used routinely because its red image caused eye strain. (DAPI and PI also indicated similar percentages of dead cells in cytotoxicity tests using isolated lymphocytes) (Tanke *et al.*, 1982).

Dow and Peacock (1989) found no leakage through goblet cell apical valves for up to 30 min after ionophoretic inoculation of the fluorescent dyes Lucifer yellow and 6-carboxy-fluorescein into cavities of goblet cells from the middle region of *Manduca sexta* midgut, although there was rapid horizontal dye movement into a few neighbouring cells. In this study, PI and DAPI did not move laterally from damaged, columnar epithelial cells into adjacent healthy cells (Figure 3.1), but were discharged apically in droplets from goblet cells. Likewise, Percy and Fast (1983) detected no lateral intercellular diffusion of toxic symptoms in *Bombyx mori* treated with B.t.k..

### 3.4.2 Control treatments on isolated midgut epithelial tissue

Midgut epithelium remained viable for up to 24 h post-dissection as determined by exclusion of trypan blue, PI and DAPI, dyes which indicate dead or damaged cells. Continuous PM synthesis and basal lamina production confirmed maintenance of some normal tissue function. Since this assay system is based on stain incorporation into cells affected by B.t. toxins, all other experimental conditions were examined as possible sources of cell injury. Addition of solubilization buffer (in equivalent amounts to that used with Cry1Ac) did not cause incorporation of dead cell indicators, so staining in columnar cells after addition of Cry1Ac was not due to the buffer used. Solubilization buffer (50 mM $Na_2CO_3$-HCl/DTT pH 10.5, applied at the same concentration as used with soluble crystals) also has no effect on a *T. ni* cell line derived from adult ovary (Thomas and Ellar, 1983). Here, *E. coli* HB-101 culture products were assessed for toxic effects because Cry1Ac crystals were produced in that bacteria, but *E. coli* cell components did not cause staining by DAPI. That result is

not surprising since *E. coli* has never been isolated as an insect pathogen (Lysenko, 1985) and it causes no mortality when injected directly into the haemocoel of *M. sexta* larvae (Dunn and Drake, 1983). In some untreated wholemount preparations the medium became contaminated with unidentified bacteria, yet cells still remained unstained by dead cell indicators.

Exposure to UV radiation did not increase fluorochrome staining in epithelial tissues. Repeated viewing of untreated tissues under UV illumination did not cause increased DAPI incorporation over time. Similarly, staining was not different in Cry1Ac-treated tissues viewed twice under UV illumination from tissues treated the same and viewed only once. Nevertheless, tissue viewing time was kept to a minimum for all wholemounts to minimize damage resulting from excitation of fluorochromes or cellular constituents excited by high intensities of UV irradiation, which are generally damaging to cells (Arndt-Jovin and Jovin, 1989). All wholemounts were stored and viewed in the dark to minimize excitation of fluorochromes.

### 3.4.3 Toxin treatments on isolated midgut epithelial tissue

Addition of Cry1Ac protoxin to midgut epithelial wholemounts resulted in uptake of both DAPI and PI into columnar epithelial cells. Both fluorochromes were excluded from untreated *T. ni* midgut columnar epithelial cells, and from cells treated with either solubilization buffer or *E. coli* HB101 culture products. I therefore concluded that solubilized protoxin was converted to active toxin by the midgut epithelial tissue, and further processing of protoxin by treatment with proteolytic

enzymes was unnecessary. It is not known if digestive enzymes were secreted after wholemount production, or if residual enzymes remained closely adherent to cell surfaces, but since the staining reaction to Cry1Ac was the same in tissues from both moulting and intermoult larvae the former seems most likely. During moulting, *T. ni* stop feeding and the midgut empties (Barbehenn and Keddie, 1992). Remaining digestive enzymes are likely lost as well. Presence of nutrients in SAHS likely stimulated epithelial cells to secrete digestive enzymes which then activated Cry1Ac protoxin. Although the pH optimum for proteolytic enzymes from the midgut of *T. ni* is high (100% maximum activity at about pH 10.25), there is 30% maximum activity in the range from pH 6.0 to 8.0 (Wolfson and Murdock, 1990). The pH of SAHS was 7.1. The ability of the epithelium to activate Cry1Ac protoxin confirms earlier observations that some normal physiological functions are retained by the tissue in this preparation.

Formation of PM, although interesting in itself, caused some problems in assessing Cry1Ac toxicity. In some wholemounts newly formed PM prevented staining of columnar cells after treatment with Cry1Ac. Once PM was removed, those cells stained within one hour. PM is synthesized by brush border microvilli along the length of the midgut in *T. ni* (Adang and Spence, 1981). It begins as an amorphous matrix, aggregates into a fibrous matrix and matures at the tips of the microvilli. In whole insects PM is not permeable to B.t. spores or vegetative cells, but it is thought that solubilized crystal proteins enter through oblique discontinuities in the membrane or after its permeability has been altered by microbial proteases (Adang and Spence,

1983). Here, PM formation began as an amorphous material produced by cells in the anterior and middle regions. Within one hour after wholemount production, an intact layer of material was present above the tissue. Examination of cells through this overlying film was sometimes difficult, as cells appeared blurred. Quantitative assessment of toxic effects would also be hampered by PM formation since ICPs bind to the PM itself: Cry1B and Cry3A both were bound to the PM in *M. sexta* although neither is toxic to the insect (Bravo *et al.*,1992b).

In general, staining reactions of cells in tissues from moulting insects were more uniform, with less extraneous cell damage than in tissues from intermoult larvae. Tissues from intermoult larvae possibly have greater cell damage due to removal of PM. PM is formed during intermoult and released by microvillar tips on columnar cells (Adang and Spence, 1981) and during its removal cells may be slightly damaged. In dissections here, PM was usually completely free of epithelial cells in the posterior and middle regions of the midgut, but often tightly adherent to cells in the anterior region. In tissues taken from moulting insects, PM was rudimentary and easily removed without causing tissue damage. In addition, basal lamina and connective tissue removal was easier in insects dissected during moulting, although slightly longer incubations were required. Tissues from insects dissected during intermoult were generally more fragile and had greater staining of damaged cells as a result. In some untreated wholemounts prepared from either moulting or intermoult larvae, staining occurred in cells along the tops of ridges. Ridges were present in fourth instar larvae (*i.e.* folds) but insignificant in the moulting larvae used to prepare wholemounts.

These ridges may have been areas in close contact with PM or may have been damaged by insufficient fluid coverage in the well. They may also indicate cells with increased permeability due to normal physiological function (*i.e.* nutrient absorption, enzyme secretion).

At each stage in their development, columnar epithelial cells, in tissues treated with Cry1Ac, showed sufficient alteration in cell membrane permeability to allow infiltration of trypan blue, PI and DAPI. This change in membrane permeability after treatment with B.t. δ-endotoxin has been noted previously: cells of *Pieris brassicae* became permeable to ruthenium red within 5 min of treatment (Ebersold *et al.*, 1978). Neighbouring goblet cells remained unstained, indicating that goblet cells remained healthy throughout.

There were more extruding columnar cells in tissues treated with Cry1Ac than in untreated tissues. In untreated tissue these extruding cells constituted ≤ 10% of the total cell population and likely represent normal cell turnover in the midgut (2.4.1). Extruding cells in untreated tissue stained with trypan blue before non-extruding cells. Numbers of extruding cells in Cry1Ac-treated tissue were higher than in untreated tissues. They also stained more rapidly than did other columnar epithelial cells similarly treated. Sublethal doses of B.t. δ-endotoxins cause columnar and goblet cell membranes to extrude (Spies and Spence, 1985). It therefore seems likely that extruding cells in Cry1Ac-treated tissues are composed of two sub-populations of columnar cells: those predestined for replacement, which may have more permeable membranes due to their degeneration, and those fully differentiated columnar cells

affected by Cry1Ac so that their membranes were now permeable to trypan blue, PI and DAPI. Fully mature columnar cells may have a larger surface area available for toxin binding, so they are affected by Cry1Ac sooner than less differentiated smaller columnar cells. Or mature cells may have a different population of toxin receptors, or receptors with slightly different conformation. Nevertheless, in wholemounts treated with Cry1Ac, all columnar epithelial cells, extruding or not, were stained with DAPI within 2 h post-treatment.

In tissues isolated from moulting insects, staining after addition of Cry1Ac was observed in basal regenerative cells earlier than in non-extruding, columnar epithelial cells. Staining was unexpected in regenerative cells, because, in whole insects, these cells are not in direct contact with the gut lumen. Tests with dead cell indicators demonstrated that regenerative cells were not damaged during tissue preparation (*i.e.* basal cells from untreated tissues remained unstained by DAPI for up to 24 h post-treatment). Uptake of these stains in the presence of Cry1Ac suggests, instead, that receptors were present on the exposed, basal surfaces of these cells. Alternatively, toxin may have reached apical and lateral surfaces of regenerative cells through gaps between epithelial cells. Immunocytochemical binding experiments have shown that B.t. ICPs bind to microvillar brush border of epithelial cells (Bravo *et al.*, 1992a, 1992b; Estada and Ferre, 1994). Aminopeptidase N has been identified as the receptor for Cry1Ac in *M. sexta* (Knight *et al.*, 1994; Sangadala *et al.*, 1994) and *Lymantria dispar* (Valaitis *et al.*, 1995). It is an endogenous apical protein. Columnar epithelial cells are polarized cells, and although sorting of apical and basolateral proteins is

highly efficient, most polarized cells secrete apical proteins from both surfaces (Pugsley, 1989), so this receptor certainly could be present on the basal surface of regenerative cells. Aminopeptidase N is anchored in the membrane of epithelial cells of *M. sexta* by glycosyl-phosphatidylinositol (GPI) (Garczynski and Adang, 1995; Knight *et al.*, 1995). The lipid GPI anchor, besides allowing for endogenous phospholipase C-catalyzed release of phosphatidylinositol lipoproteins from the cell surface, also allows greater lateral mobility in the membrane than a peptide anchor would (Pugsley, 1989), and as cells differentiate, perhaps receptors on the basolateral surfaces migrate to the apical surface.

A colour change from green to orange occurred in goblet cell cavities in tissues treated with Cry1Ac and stained with AO. Acridine orange is a metachromatic dye that exists in two different complexes: as a monomer in low concentrations it fluoresces at 535 nm (green), and as a polymer at higher concentrations the emission peak is 660 nm (red) (West, 1969). Two complexes are formed with nucleic acids: at low concentrations a stable complex is formed with emission at 540 nm, whereas at intracellular dye concentrations higher than $5 \times 10^{-4}$ M the second complex, which is very sensitive to fluorescence-exciting radiation, forms (West, 1969; Pace and Sachs, 1982). Cells exhibiting cytoplasmic reddening undergo an irreversible deterioration which continues even in the dark. Acridine orange also concentrates at anionic sites. In a model proposed by Knowles (1994; Knowles and Dow, 1993) to explain action of B.t. toxins on lepidopteran gut, non-selective pores formed in columnar cell membranes cause entry of $K^+$ and efflux of $H^+$. The resultant membrane

depolarization and rise in intracellular pH probably lead to closure of gap junctions, which isolates goblet cells from columnar cells. Goblet cells then stop active $K^+$ transport as the $K^+/H^+$ exchanger activity decreases, which in turn inactivates V-ATPase, causing an increase in pH in the goblet cavity. This increased pH is reflected by a colour shift in AO staining from green to red. Pace and Sachs (1982) found a similar colour change in insulin-containing secretory granules as addition of glucose induced a colour change from yellow-green to red. Interestingly, Fast and Donaghue (1971) report that glucose uptake is stimulated within 1 min of administration of B.t. δ-endotoxin to *B. mori* larvae, however that occurs in columnar, not goblet, cells. Dow (1992) found no such incorporation of acridine orange in goblet cell cavities in isolated lepidopteran midgut. He found that under normal conditions in actively pumping midgut, AO is excluded from cavities, and red staining is present only when the ion transport capacity of the cavity has been compromised. In these preparations, cavity colour change from green to red on addition of Cry1Ac indicated a change in its transport capacity. Trypan blue was excluded from goblet cavities in enzymatically-untreated midguts but was present in cavities in wholemounts prepared with dispase (2.3.2). In other words, there was no access of vital dyes into the goblet cell through the apical opening or laterally from columnar cells or from the basal surface. Once the basal surface of the cell was exposed to bathing medium after dispase removal of the basal lamina, dyes may have entered goblet cells through channels in the basal membrane and were pumped through the cavity and out the apical valve. Dow (1992) also noted that isolated goblet cells stained red with AO,

and concluded they were functionally dead. Here, droplets were seen arising from goblet cells in untreated tissue up to 9 h after wholemount production, indicating continued activity of goblet cell pumps. It could be argued that removal of the basal lamina and connective tissue damages goblet cells, thereby allowing AO accumulation into their cavities, however in tissues bathed in SAHS and AO, cavities are dark or green, not red, as would be the case if goblet cells were 'dead' or non-functional. Also, addition of low concentrations of AO (2.5 μm) to tissues bathed only in SAHS resulted in both green and red droplets in the media, indicating that goblet cells sequestered enough AO to allow formation of the complex which fluoresces at 660 nm.

Wholemount tissues produced here are only one cell layer thick, and without additional support are too fragile to suspend over a cavity to allow potential difference measurements in the usual fashion (Dow, 1992). Use of specific fluorescent probes may help detect goblet cell function.

In midgut epithelial wholemounts, columnar epithelial cells stained with DAPI if either purified inclusion bodies or Cry1Ac protoxin was used. This indicated tissues were still capable of some normal physiological functions. Hofmann *et al.* (1988) report that BBMVs from *M. sexta* can proteolytically process protoxin from CryIA(b). This may be possible because crystals have some endogenous proteolytic enzymes adsorbed on their surface or enclosed within the crystal lattice (Chestukhina *et al.*, 1978). There are no reports of proteinases in crystals produced in *E. coli.*

Addition of more than 1.0 μg of Cry1Ac protoxin resulted in complete tissue

disintegration whereas addition of between 50 and 400 ng resulted in staining in most columnar cells and the wholemounts remained intact. Very few cells were stained when 5 ng of Cry1Ac protoxin was added to the wholemounts but because tissues were very fragile it was difficult to ensure adequate mixing of fluids in media wells.

### 3.4.4 Insect bioassays

#### 3.4.4.1 Oral inoculation bioassay

Results from the inoculation bioassay of Cry1Ac protoxin indicated increased mortality correlated with amount of food consumed before inoculation. Inoculation with Cry1Ac protoxin did not cause greater mortality in unfed insects than in controls, suggesting that unfed insects were less susceptible to toxin. Since most phytophagous Lepidoptera have a lowered midgut pH during starvation, (Heimpel, 1955; 1961) it is likely midgut conditions (both pH and enzyme activity) in unfed *T. ni* were not suitable to activate Cry1Ac protoxin. Consumption of exuvium alone was sufficient to increase mortality rates (remnants of the cast exuviae were still present in midguts at dissection). Highest mortality was observed in insects fed diet before inoculation with Cry1Ac. Although those larvae were without food for 2 h before inoculation, food was still present in the midgut. Consumption of any amount of food material likely increased midgut pH and stimulated production of digestive enzymes which activated Cry1Ac protoxin.

Mortality in control insects was due to physical damage (*i.e.* gut stretching), or to other adverse effects of the inoculation technique itself. These factors were accounted for in treated insects by using Abbott's formula.

### 3.4.4.2 Oral inoculation followed by midgut assay

Numbers of insects used in this experiment were limited, yet some general conclusions can be inferred from the results. Staining results from epithelial tissues prepared from whole insects inoculated with solubilization buffer or Cry1Ac protoxin were consistent with staining observed after DAPI addition to wholemounts treated with SAHS and solubilization buffer. DAPI staining did not occur in columnar epithelial cells (other than extruding cells) in any wholemounts prepared from insects inoculated with solubilization buffer, but did occur in damaged cells along cut edges and transitorily in goblet cell cavities.

Wholemounts were prepared 4 h post-inoculation from fed larvae inoculated at 20 h post-moult with either SAHS or Cry1Ac. Columnar epithelial cells were stained by DAPI only in the Cry1Ac-treated insect. In contrast, there was no DAPI staining in columnar cells in wholemounts prepared 27 or 28 h post-inoculation from fed larvae inoculated with SAHS or Cry1Ac more than 24 h after the moult. This implies that cells were able to recover sometime between 4 and 27 h post-inoculation. Eighty percent of inoculated insects survived, indicating that the dose used here on fourth instar larvae (0.95 µg solubilized Cry1Ac protein) was not lethal. When *M. sexta* is treated with a sub-lethal dose of B.t., cell division is stimulated within 20 h after recovery and full recovery is complete within 48 h (Spies and Spence, 1985). Here, feeding inhibition was observed in the first 24 h post-inoculation, and insects then recovered.

This was not the case in wholemounts treated directly with Cry1Ac: columnar

epithelial cells were stained by PI or DAPI when stain was added more than 15 h after toxin treatment. (Wholemounts had been treated with Cry1Ac, rinsed with SAHS and stained 15 to 27 h later.) Staining likely was due either to continued presence of unbound toxin in the media (tissues were too fragile to permit vigorous rinsing with SAHS) or membrane permeability was still affected by earlier binding of Cry1Ac.

Individual cell recovery did not occur in wholemount preparations, and it is unlikely to occur in these tissues without additional nutritional input. DAPI binds irreversibly to nuclear DNA, so even if cell membrane permeability had returned to normal, cells would remain stained and therefore be classified as damaged. Columnar epithelial cells did slough off apically into the bathing media, and that is one step in tissue recovery.

There was no DAPI uptake into columnar cells in the posterior region of the midgut in any wholemounts prepared from insects after inoculation of Cry1Ac protoxin although columnar cells in the posterior region were stained following addition of Cry1Ac directly to wholemounts. This indicated that receptors were present on columnar cells in that region. Changes in blood levels of $K^+$ after inoculation of B.t. toxins to ligatured *B. mori* larvae demonstrate that although the anterior third of the midgut is the primary site of action of B.t. toxins (Heimpel and Angus, 1959) the posterior end is equally capable of mediating delta-endotoxin effects (Nishiitsutsuji-Uwo and Endo, 1980). Heimpel and Angus (1959) also showed pathological effects from B.t. when it was placed anywhere in the midgut in *B. mori*. Bravo *et al.* (1992b) suggest inhibition of midgut motility or unequal distribution of

receptors as the reason for preferential retention of Cry1Ab and Cry1B proteins in the anterior region of the midgut in *M. sexta* and *P. xylostella*. Here, fed insects were inoculated from 12 h to more than 24 h post-moult, after PM formation was complete. In *M. sexta*, posterior midgut columnar epithelial cells are not thought to secrete digestive enzymes (Cioffi, 1980). If this is true for *T. ni*, perhaps lack of staining in columnar cells in the posterior region is due to containment of digestive enzymes through regional compartmentalization in the insect midgut. Conversely, fluid mixing occurs in the bathing medium surrounding epithelial wholemount tissues so that proteolytically-activated toxin reached columnar cells in the posterior region in these tissue preparations.

It was not possible to prepare a wholemount from an unfed larva 3 h after inoculation with Cry1Ac. This insect did not feed after the inoculation and its gut was very shrunken. By 14 h post-inoculation, another larva had eaten and a wholemount was prepared easily from it. This condition was not seen in those unfed insects inoculated with SAHS (personal observation, data not shown), evidence that inoculated larvae suffered from feeding inhibition after Cry1Ac treatment.

### 3.5 Conclusions

In summary, midgut epithelium dissected from moulting or intermoult *T.* ni larvae was maintained as a flat sheet for up to 24 h post-dissection in an artificial haemolymph solution. Tissues were given various treatments, including Cry1Ac protoxin, and cells affected by these were identified by vital dye staining. Consistent staining results were obtained with the fluorochromes PI and DAPI: both dyes were

excluded from columnar epithelial cells in untreated midgut tissue for up to 24 h post-dissection. These same dyes entered regenerative and columnar epithelial cells in tissues treated with Cry1Ac. I speculate that channels opened or pores formed in response to Cry1Ac binding to receptors on columnar epithelial cells allowed accumulation of PI and DAPI, permitting easy identification of target cells. While cells were not necessarily 'dead', they showed sufficient alteration in membrane permeability to allow infiltration of dyes normally excluded from healthy cells. Membrane permeability was compromised in regenerative, differentiating, and fully differentiated columnar epithelial cells in all regions of the midgut of *T. ni* in epithelial tissue wholemounts treated with toxin, whereas toxin did not affect permeability in cells from the posterior regions of the midgut in whole insects inoculated directly with Cry1Ac.

Most commercial formulations of B.t. consist of several genes (*eg. B. thuringiensis* var. *kurstaki* (Dipel, Abbott; MVP, Mycogen) contains cry1Aa, cry1Ab, cry1Ac, cry2A and cry2B protoxin genes (Höfte and Whiteley, 1989; Aronson *et al.*, 1991; Charnley, 1991). In this study, toxin produced from only a single gene (cry1Ac) was used. There may be differences in targeted cells or in cell response due to each gene product, and these differences could be reflected by differential cell staining using this technique. Additional research has been conducted to determine response of *T. ni* epithelial tissue wholemounts to other Cry1A toxins, Cry1C, Cry1E, Cry2A and Cry3A.

Figure 3.1 Posterior region of midgut from *T. ni* larva moulting to fourth instar, treated with SAHS and stained with DAPI. Note stained nuclei (arrow) in damaged columnar epithelial cells around hole in tissue (U1 filter plus red and blue daylight filters). Scale bar = 25 μm

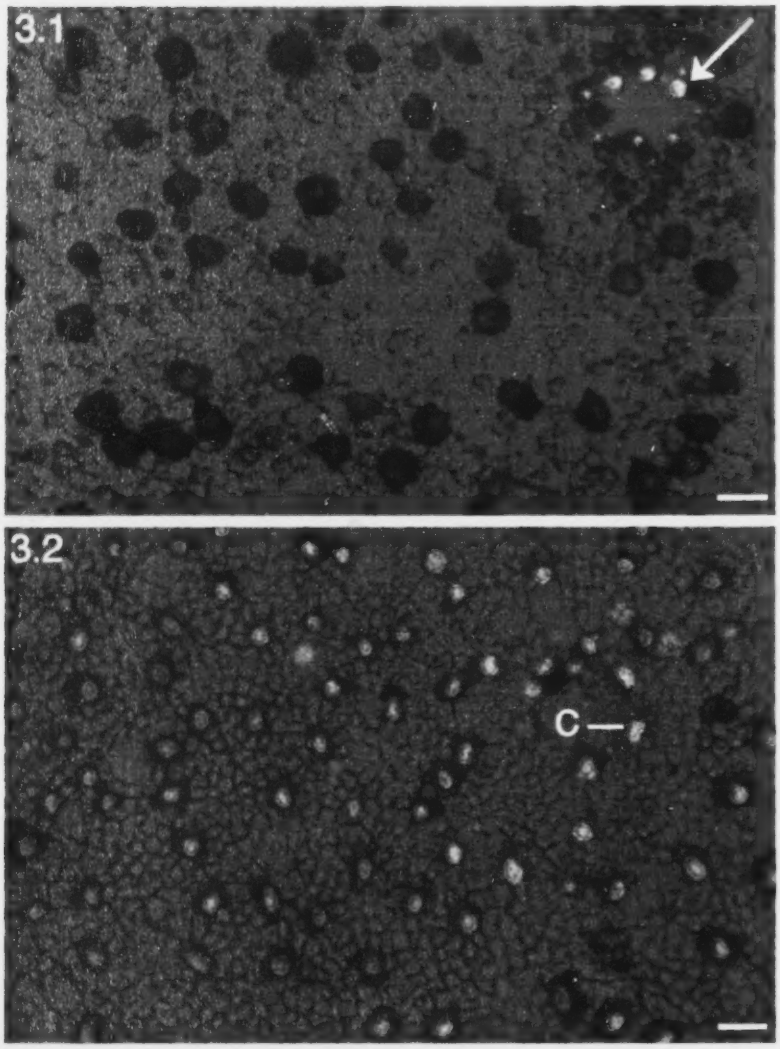


Figure 3.2 Posterior region of midgut from fourth instar *T. ni* larva 24 h after additon of solubilized Cry1Ac and stained with DAPI. Note staining of fully differentiated columnar epithelial cells (C). (U1 filter plus red and blue daylight filters). Scale bar = 25 μm

Figure 3.3 Epithelial wholemount from fourth instar *T. ni* larva treated with SAHS and stained with 50 µM AO and 25 µM PI. Note columnar cell (C), goblet cell (G), and differentiating cell (D) (U1 filter). Scale bar = 25 µm.

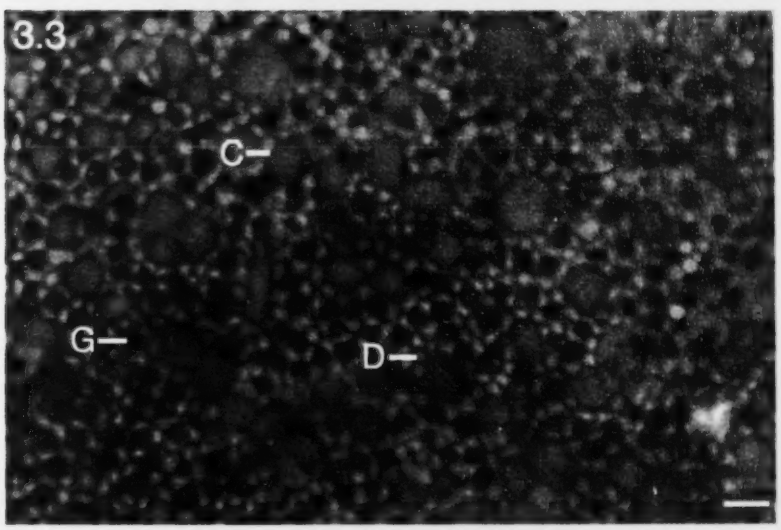

Figure 3.4 Columnar epithelial wholemount from the posterior region of a newly moulted fourth instar *T. ni* larva stained with 25 µm PI and 50 µm AO (U1 filter). Note cells in nest (N). Scale bar = 25 µm.

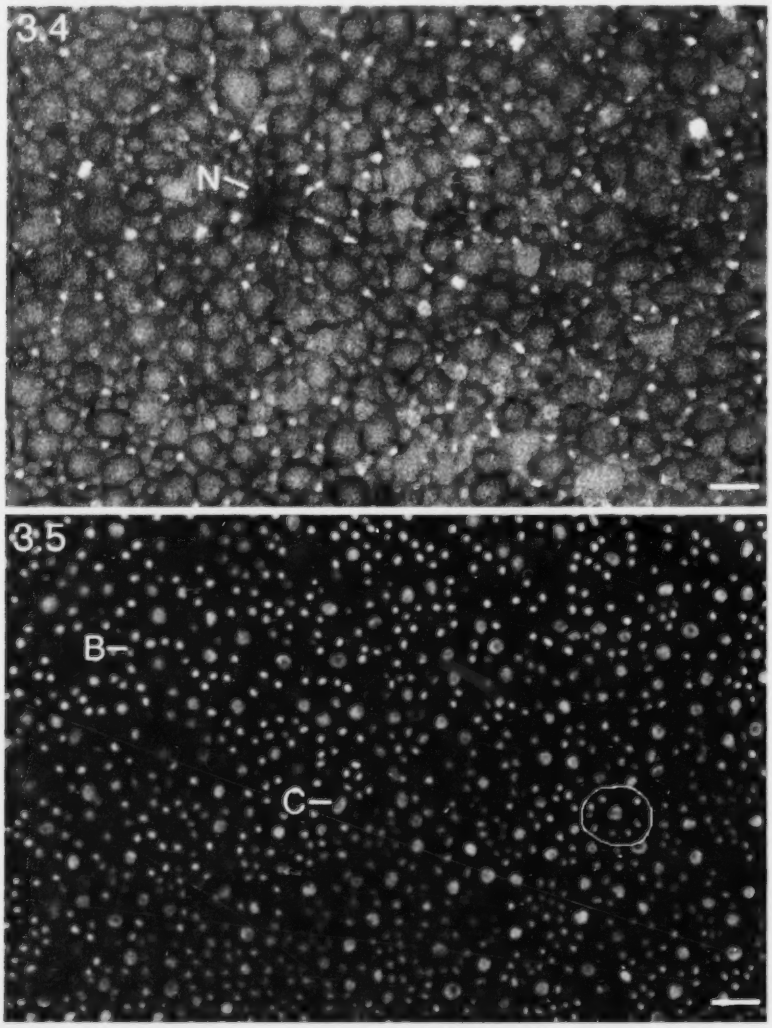


Figure 3.5 Epithelial wholemount from fourth instar *T. ni* 24 h after treatment with Cry1Ac and stained with DAPI. Note circle demonstrating pattern of stained basal cells (B) around stained columnar epithelial cells (C) (U1 filter). Scale bar = 25 µm.

Figures 3.6 - 3.7 Midgut epithelium wholemounts from middle region of third instar *T. ni* larvae treated with solubilized Cry1Ac and stained with 50 μM PI and 50 μM AO. Columnar cell (C); goblet cell (G); (U1 filter). Scale bar = 25 μm.

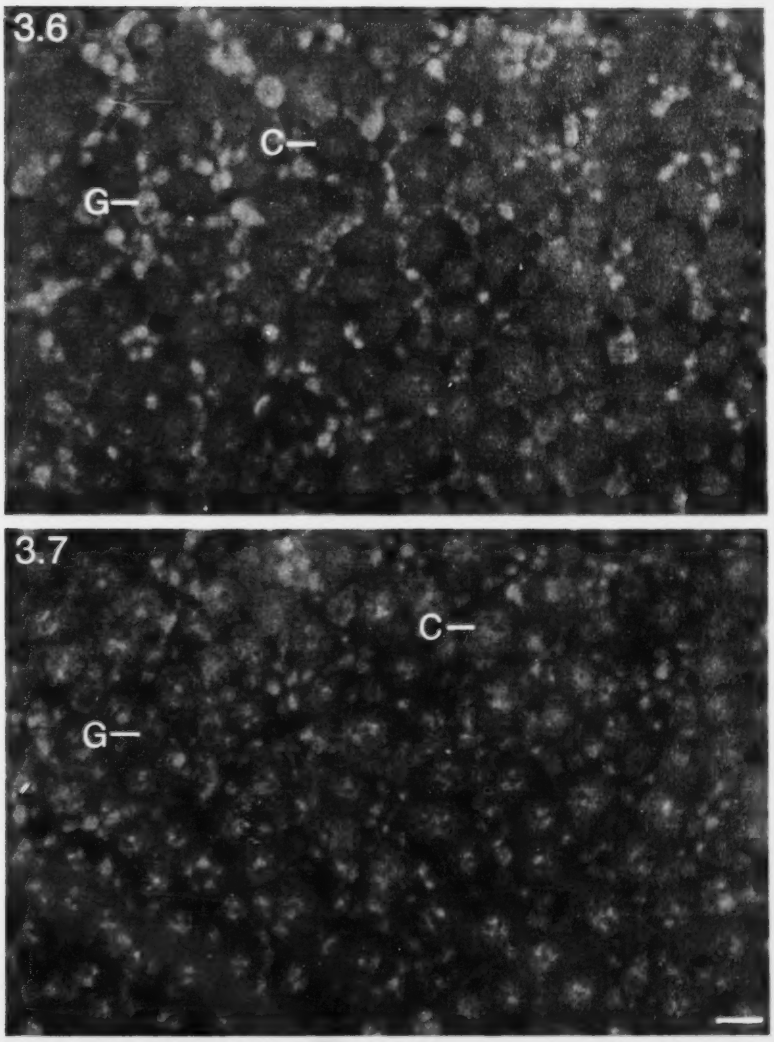


Figure 3.6 Micrograph taken 40 min after addition of Cry1Ac protoxin.
Figure 3.7 Same field as Figure 3.6 taken 60 min after protoxin addition.

Figure 3.8 Epithelial wholemount from *T. ni* 24 h after additon of Cry1Ac protoxin. Note 'footprint' of basal lamina (BL) (DYLT filter). Scale bar = 25 μm.

Table 3.1. Colour of vital stains in cells

| | | | LIVING CELLS | | DEAD/DYING CELLS | | |
|---|---|---|---|---|---|---|---|
| STAIN | FILTER | CELL TYPE | CYTOPLASM | NUCLEUS | CYTOPLASM | NUCLEUS | REFERENCE |
| AO | U1 | mammalian | green | green | yellow-orange | yellow-orange | West 1969 |
| PI | U1 | lymphocytes | unstained | unstained | red | red | Tanke et al. 1982 |
| PI + AO | U1 | pancreatic islet cells | green | green | red | red | Bank 1987 |
| AO + PI | U1 | pancreatic islet cells | green | green | yellow-orange | red | Bank 1987 |
| DAPI | U1 | lymphocytes | unstained | unstained | unstained/blue | blue | Tanke et al. 1982 |
| TRYPAN BLUE | DYLT | HeLa cells | unstained | unstained | blue | blue | Tennant 1964 |



| | |
|---|---|
| AO | Acridine orange |
| PI | Propidium iodide |
| DAPI | 4',6-Diamidino-2-phenylindole |
| U1 | Ultraviolet filter, BP 330-380, epiillumination |
| DYLT | Daylight filter, transmitted light |

Table 3.2. Comparison of third instars of *T. ni* inoculated with SAHS or 0.4 µg Cry1Ac protoxin.

| Treatment | n | Mean Larval Weight (mg) Start (mean ± S.D.) | n | after 5 d (mean ± S.D.) | n | Mean Pupal Weight (mean ± S.D.) | No. Days to Pupation (mean ± S.D.) | No. Days to Eclosion (mean ± S.D.) | n | Mortality | Abbott's Corrected Mortality |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SAHS - Group I | 13 | 5.8 ± 2.0 | 11 | 214.9 ± 68.2 | 10 | 248.7 ± 16.9 | 7.9 ± 0.7 | 13.9 ± 0.7 | 3 | 23% | |
| SAHS - Group II | 12 | 5.7 ± 1.7 | 12 | 196.3 ± 58.5 | 11 | 238.0 ± 31.3 | 7.9 ± 0.3 | 13.9 ± 0.3 | 2 | 17% | |
| SAHS - Group III | 11 | 17.1 ± 5.6 | 6 | 276.2 ± 91.0 | 9 | 243.6 ± 21.2 | 6.9 ± 1.2 | 12.9 ± 1.2 | 2 | 18% | |
| Cry1Ac - Group I | 13 | 4.7 ± 1.8 | 10 | 174.1 ± 64.6 | 10 | 244.9 ± 23.2 | 8.5 ± 0.8 | 14.6 ± 1.1 | 3 | 23% | 0% |
| Cry1Ac - Group II | 7 | 5.1 ± 2.1 | 5 | 197.2 ± 81.3 | 5 | 235.9 ± 21.0 | 8.4 ± 1.1 | 14.8 ± 1.9 | 2 | 29% | 14% |
| Cry1Ac - Group III | 10 | 19.0 ± 6.8 | 5 | 248.9 ± 60.6 | 5 | 231.0 ± 18.8 | 7.3 ± 1.0 | 13.3 ± 1.0 | 6[1] | 60% | 51%** |

Group I unfed; did not eat cast exuvium or diet
Group II unfed; ate cast exuvium only
Group III fed; ate artificial diet (± cast exuvium)
SAHS sterile artificial hemolymph solution
moulting larvae were not weighed
[1] one pupa died
** denotes significance at 0.01 using Chi-square
Larvae were reared at 27°C ± 2°C

Table 3.3 DAPI staining in epithelial cells from wholemounts prepared after inoculation with 0.95 µg CryIAc protoxin or 5 µl solubilization buffer into fed or unfed *T. ni* larvae.

| Pre-inoculation Treatment | Inoculation Treatment | Time of Inoculation Post-moult (h) | Time Whole Mount Prepared ost-inoculation (h | First Observation Time Post-inoculation (h) | Cell Types | | | | Last Observation Time Post-inoculation (h) | Cell Types | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | edg | col | ext | gob | | edg | col | ext | gob |
| fed | buffer | 20 | 4 | 6 | + | - | ± | - | 20 | + | - | ± | |
| fed | buffer | > 24 | 4 | 9 | + | - | ± | [+] | nd | | | | |
| fed | buffer | > 24 | 27 | 30 | + | - | - | [+] | nd | | | | |
| fed | CryIA(c) | 20 | 4 | 6 | + | + | ± | - | 20 | + | + | ± | - |
| fed | CryIA(c) | 12 | 3 | 9 | + | + | + | [+] | 28 | + | + | + | [+] |
| fed | CryIA(c) | > 24 | 6 | 9 | + | - | - | [+] | 27 | + | - | ± | [+] |
| fed | CryIA(c) | > 24 | 28 | 30 | + | - | ± | - | nd | | | | |
| unfed | CryIA(c) | 14 | 3 | np | | | | | nd | | | | |
| unfed | CryIA(c) | 14 | 27 | 30 | + | - | - | [+] | nd | | | | |

edg damaged cells along cut edges
col columnar epithelial cells
ext extruding columnar epithelial cells
gob goblet cells
nd not done
np could not prepare wholemount
\+ uptake into cells
± uptake into some but not all cells
\- no uptake into cells
[+] stain uptake confined to goblet cavity; may be transitory

NOTE: With the exception of extruding columnar epithelial cells, no DAPI staining occurred in cells in the posterior region of the midgut in any preparation.

Table 3.4 Colour reactions of vital stains in columnar epithelial cells treated with SAHS. (Tissues observed between 5 min and 24 h post-dissection).

| | | HEALTHY CELLS | | DAMAGED CELLS | |
|---|---|---|---|---|---|
| STAIN | conc.(µM) | CYTOPLASM | NUCLEUS | CYTOPLASM | NUCLEUS |
| AO | 10 | green | green | green | green |
| | 50 | green | green | orange | orange |
| | 100 | green | green | orange | orange |
| PI | 10 | unstained | unstained | red | red |
| | 25 | unstained | unstained | red | red |
| | 75 | unstained | unstained | red | red |
| AO + PI | 2.5,75 | green | green | red | red |
| | 5,10 | green | green | red | red |
| | 10,25 | green | green | red | red |
| | 25,75 | green | green | red | red |
| | 50,25 | green | green | red | red |
| | 50,50 | green | green | red | red |
| TRYPAN BLUE | 0.01 - 0.02% | unstained | unstained | blue | blue |
| DAPI | 100 µM | unstained | unstained | none/pale blue | blue |

AO Acridine orange
PI Propidium iodide
DAPI 4',6-Diamidino-2-phenylindole

Table 3.5 Vital dye uptake in midgut epithelial cells treated with SAHS or Cry1Ac protoxin.

| | SAHS | | | | | Cry1Ac protoxin | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| STAIN | edg | col | ext | gob | bas | edg | col | ext | gob | bas |
| AO 50 µM | + | + | + | [+] | + | + | + | + | [+] | + |
| PI 50 µM | + | - | ± | [+] | - | + | + | + | [+] | + |
| Trypan blue 0.02% | + | - | ± | [+] | - | + | + | + | [+] | + |
| DAPI 1 µM | + | - | ± | [+] | - | + | + | + | [+] | + |

| | |
|---|---|
| edg | dead or damaged cells along cut edges |
| col | columnar epithelial cells |
| ext | extruding columnar epithelial cells |
| gob | goblet cells |
| bas | small basal cells - presumably regenerative |
| + | stain uptake into cell |
| - | no stain uptake into cell |
| ± | stain uptake into some cells |
| [+] | stain uptake confined to goblet cavity; transitory |

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## Chapter 4 - Use of a new midgut epithelial tissue assay to identify *Bacillus thuringiensis* proteins toxic to *Trichoplusia ni*

### 4.1 Introduction

*Bacillus thuringiensis* (B.t.) is a gram-positive bacterium that produces crystalline inclusions during sporulation. After sporulation the cell lyses to release a spore and a crystal which has insecticidal properties. Often the insecticidal crystal protein (ICP) is a protoxin which, when degraded by proteolytic enzymes in the insect midgut, forms smaller toxic peptides called endotoxins (Höfte and Whiteley, 1989). Susceptible insects have high midgut pH, or specific reducing substances, or both that play a role in crystal dissolution (Cooksey, 1971). Activated toxin reacts with epithelial cells of susceptible insects by binding to brush border membrane receptors of midgut epithelium (Heimpel and Angus, 1959; Fast and Angus, 1965; Ebersold *et al.*, 1977; Tojo, 1986; Hofmann *et al.*, 1988; Van Rie *et al.*, 1990). Recently, aminopeptidase N has been identified as a receptor for one toxin, Cry1Ac (Knight *et al.*, 1994; Sangadala *et al.*, 1994; Valaitis *et al.*, 1995). The exact mechanism of toxicity is still unknown, but it is thought the toxin binds to a receptor in the membrane of susceptible cells and creates a pore (Knowles and Ellar, 1987). The flow of ions and accompanying water into the cell causes swelling and cell lysis.

Höfte and Whiteley (1989) first proposed a nomenclature scheme for B.t. toxins based on their amino acid sequence and host range (CryI to CryIV). Crickmore *et al.* (1995) updated the classification relying solely on amino acid sequence (Cry1 to Cry15). Feitelson *et al.* (1992) constructed an evolutionary tree based on amino acid sequence

comparisons to demonstrate relationships between various classes of Cry proteins. By definition, toxins within one class (*e.g.* Cry1) should have similar host specificity. Because they also have similar amino acid sequences, the proteins they produce theoretically should have similar properties and behave in similar ways. Toxins within any subclass (*e.g.* Cry1A) are even more closely related and should produce similar toxic reactions *in vivo* and *in vitro*. In fact Cry1A proteins have overlapping activity spectra (Höfte and Whiteley, 1989).

In previous chapters, I described development of a midgut epithelium tissue assay used to identify cells affected by a single protein produced by B.t.: the δ-endotoxin Cry1Ac. Several Cry proteins will be used here in toxicity assays on *Trichoplusia ni*, the cabbage looper. Toxins will be added to isolated midgut epithelium tissue wholemounts along with the fluorochrome DAPI (4',6-diamidino-2-phenylindole). DAPI is normally excluded from healthy cells (Taylor and Milthorpe, 1980; Tanke *et al.*, 1982). Its incorporation into epithelial cells will identify those cells whose membrane permeability has been altered through pore formation or other membrane perturbations. Only single-gene products will be used here: Cry1Ac, Cry1Ab, Cry1B, Cry1C, Cry1E, Cry2A and Cry3A. All but Cry3A are considered active against Lepidoptera.

## 4.2 Materials and methods

### 4.2.1 Insects

*Trichoplusia ni* larvae were maintained at 26°C ± 1°C under a 14L:10D photoperiod and fed on artificial diet containing antibiotics (2.2.1). Third and fourth instars, including moulting larvae, were selected for preparation of epithelial

wholemounts. Under these rearing conditions most larvae completed moulting between 18:00 and 21:00. Some larvae were closely monitored and assigned time zero after moulting when they started to consume cast exuviae.

Epithelial tissue wholemounts were prepared as described previously (2.2.2). Tissues were treated with B.t. toxins, stained with the fluorochrome DAPI and examined under UV epillumination up to three times post-treatment. Wholemounts also were left untreated (in SAHS) or treated with solubilization buffer ($Na_2CO_3$-HCl/DTT pH 9.5); these tissues were stained with DAPI and examined under UV epillumination.

### 4.2.2 Midgut epithelial wholemounts treated with B.t. δ-endotoxins

#### 4.2.2.1 Cry1Ac

The midgut epithelial tissue wholemount assay system designed to screen for efficacy of B.t. toxins was developed using Cry1Ac provided by L. Masson (designated hereafter as Cry1Ac-LM). Cry1Ac-LM was produced as described previously (Appendix 4.1) and insoluble inclusions or solubilized protoxin were applied to epithelial tissue wholemounts along with DAPI.

Another strain containing a cloned gene for the single toxin Cry1Ac was supplied as a lyophilized spore-crystal mixture by K. Hendrickx (Ciba-Geigy Corporation) (hereafter designated as Cry1Ac-KH). Cry1Ac-KH powder (10 mg) was solubilized in 50 mM sodium carbonate/HCl pH 9.5 and 10 mM dithiothreitol (DTT) for 60 min at 37°C (Huber *et al.*, 1981). Insoluble material was removed by centrifugation at 10,000 *g* for 3 min; protoxin was retained in the supernatant. Protoxin protein concentration was determined by the Bradford method (0.32 mg/ml). Aliquots were added to midgut

epithelial wholemounts prepared from larvae moulting to fourth instar (n=3). Wholemounts were stained with DAPI, and observations were made up to 3½ h post-treatment.

#### 4.2.2.2 Cry1Ab

Cry1Ab was supplied as a lyophilized spore-crystal mixture from a single toxin wild type strain by K. Hendrickx. Cry1Ab powder (10.3 mg) was solubilized in 50 ml $Na_2CO_3$-HCl/DTT pH 9.5 at 38°C for 60 min. Insoluble material was removed by centrifugation at 10,000 *g* for 3 min; protoxin was retained in the supernatant. Protein concentration of protoxin was determined by the Bradford method (0.277 mg/ml). Aliquots of 0.55, 1.11, and 2.77 µg protein were added to midgut epithelial wholemounts prepared from fourth instar larvae (n=8) and from larvae moulting to third (n=6) and fourth instar (n=25). Observations were made for up to 8 h post-treatment.

#### 4.2.2.3 Cry1B

Cry1B was supplied as a lyophilized spore-crystal mixture from a single toxin wild type strain by K. Hendrickx. Cry1B powder (10 mg) was solubilized in 50 ml $Na_2CO_3$-HCl/DTT pH 9.5 at 38°C for 60 min. Insoluble material was removed by centrifugation at 10,000 *g* for 3 min; protoxin was retained in the supernatant. Protein concentration of protoxin was determined by the Bradford method (0.33 mg/ml). Aliquots of 0.65, 1.30, and 3.26 µg protein were added to midgut epithelial wholemounts prepared from third (n=4) and fourth (n=2) instar larvae and from larvae moulting to third (n=1) and fourth instar (n=4). Observations were made for up to 5 h post-treatment.

#### 4.2.2.4 Cry1C

Cry1C from two different sources was assayed using the midgut epithelial wholemount assay system. L. Masson provided a *cry*⁻ B.t. strain to which the cloned Cry1C recombinant had been added (Cry1C-LM). Cry1C-LM was produced as described in Appendix 4.2. A portion of the crude spore-crystal preparation was solubilized in $Na_2CO_3$-HCl/DTT at pH 9.5 for 60 min at 37°C, and solubilized protoxin was recovered in the supernatant after centrifugation. Aliquots of the spore-crystal suspension and solubilized protoxin each were added to one midgut epithelial wholemount prepared from insects moulting to the fourth instar (protein concentration not determined).

Cry1C was also supplied as a lyophilized spore-crystal mixture of a strain containing a cloned gene for the single toxin by K. Hendrickx (Cry1C-KH). Cry1C-KH powder (10 mg) was solubilized in $Na_2CO_3$-HCl/DTT pH 9.5 at 38°C for 60 min. Insoluble material was removed by centrifugation at 10,000 *g* for 3 min; protoxin was retained in the supernatant. Protein concentration of protoxin was determined by the Bradford method (0.16 mg/ml). Aliquots of 0.80, 1.59 and 3.98 µg protein were added to midgut epithelial wholemounts prepared from third instar larvae (n=10) and 0.80 µg protein was added to one wholemount prepared from a larva moulting to third instar.

#### 4.2.2.5 Cry1D

Cry1D was supplied as a lyophilized spore-crystal mixture of a strain containing a cloned gene for the single toxin by K. Hendrickx. Cry1D powder (10 mg) was solubilized in 50 ml $Na_2CO_3$-HCl/DTT pH 9.5 at 38°C for 60 min. Insoluble material was removed by centrifugation at 10,000 *g* for 3 min; protoxin was retained in the

supernatant. Protein concentration of protoxin was determined by the Bradford method (0.35 mg/ml). Aliquots of 0.70, 1.40, and 3.51 µg protein were added to midgut epithelial wholemounts prepared from third (n=4) and fourth (n=1) instar larvae and from larvae moulting to third (n=1) and fourth instar (n=4). Observations were made for up to 5½ h post-treatment.

#### 4.2.2.6 Cry1E

Cry1E was obtained from L. Masson as a recombinant added to a *cry*⁻ B.t. strain and toxin was produced as described in Appendix 4.3. Protein concentration of purified crystals was determined using the Bradford method with BSA as the standard (6.27 mg/ml). Cry1E purified crystals were solubilized in $Na_2CO_3$-HCl/DTT pH 10.0 at 37°C for 60 min. Insoluble material was removed by centrifugation at 10,000 *g* for 3 min; protoxin was retained in the supernatant. Protein concentration of protoxin was determined by the Bradford method (0.60 mg/ml).

Aliquots of purified crystals were added to midgut epithelial wholemounts prepared from third (n=1) or fourth (n=3) instar larvae and from larvae moulting to third and fourth instar (n=3 each). Solubilized protoxin was added to wholemounts prepared from a fourth instar larva and from larvae moulting to fourth instar (n=6).

#### 4.2.2.7 Cry2A

Purified Cry2A inclusion bodies (58.75 mg/ml) were provided by W. Moar, Auburn University. Inclusion bodies were diluted in SAHS and added (0.65 - 2.61 µg protein) to midgut epithelial tissue wholemounts prepared from moulting (n=1) and third instar (n=6) larvae. DAPI was added and tissues were examined for up to 6½ h post-treatment.

Cry2A inclusion bodies were solubilized in NaOH pH 11.4 for 3 h at 50°C; insoluble material was sedimented by centrifugation. Cry2A protoxin was added to midgut epithelium wholemounts from moulting *T. ni* larvae (n=4) along with DAPI and tissues were examined for up to 4⅓ h post-treatment.

#### 4.2.2.8 Cry3A

Purified Cry3A protoxin and *E. coli* MV1190 cells containing the expression plasmid for Cry3A were supplied by C. Charlton. Growth conditions and purification procedures are described in Appendix 4.4. Cry3A protoxin was diluted in SAHS and added to midgut epithelial tissue wholemounts prepared from third (n=1) or fourth (n=10) instar larvae and from larvae moulting to fourth instar (n=5). Tissues were stained with DAPI and observations were made for up to 8 h post-treatment.

### 4.2.3 Insect bioassays

#### 4.2.3.1 Cry1Ab feeding bioassay

Larvae were selected after each had shed its exuvium while moulting to fourth instar (larvae may have eaten cast exuviae). Aqueous solutions of Cry1Ab lyophilized spore-crystal powder (50, 100, 200 and 332 ng protein) were applied to disks (5µl per disk) of artificial diet (without antibiotics). Sterile water was applied to diet disks for control insects. Insects were placed individually in plastic cups with one disk. After each had eaten the entire disk individual larvae were transferred to a new cup containing artificial diet (with antibiotics). Insects were monitored daily until eclosion or death. Necropsies were performed on each dead insect to identify pathogens present. Data were analysed using probit analysis (Finney, 1971) after correction for control mortality with

Abbott's formula (Abbott, 1925).

#### 4.2.3.2 Cry1E feeding bioassay

Larvae were selected after each had shed its exuvium while moulting to fourth instar and isolated as individuals without food for 1 to 3 h (larvae may have eaten cast exuviae). Aqueous solutions of Cry1E purified crystals were applied to disks (5µl per disk) of artificial diet (without antibiotics) to deliver eight concentrations of between 10 and 300 ng protein per insect. Thirty to fifty insects were treated at each dosage. Sterile water was applied to diet disks for control insects. After insects had eaten the entire disk each was transferred to a new cup containing artificial diet (with antibiotics). Insects were monitored daily until eclosion or death. Data were analysed using probit analysis after correction for control mortality with Abbott's formula.

#### 4.2.3.3 Cry1E oral inoculation bioassay

Larvae were selected after the moult to fourth instar and divided into groups as follows: group I larvae were isolated immediately to prevent each from feeding on its cast exuvium, group II larvae were allowed to eat cast exuviae only, and group III larvae were allowed to eat cast exuviae and were supplied with artificial diet. Larvae from groups I and II were isolated individually in plastic cups to prevent cannibalism while 10 to 15 larvae from group III were kept per cup with artificial diet provided *ad libitum*. Larvae within each treatment were inoculated at the same time post-moult: larvae from groups I and II were kept without food for 18 to 20 h, then weighed and inoculated with SAHS within 2 h; larvae from group III were fed for 18 to 20 h, then isolated as individuals, weighed, and kept without food for 2 h before inoculation. Larvae were

inoculated *per os* (Keddie and Volkman, 1985) with 2 µl of Cry1E crystals containing 4 ng protein or 2 µl SAHS (control). Larvae were monitored daily until eclosion; final pupal weight was recorded 24 h after the pupal moult.

#### 4.2.3.4 Cry2A oral inoculation bioassay

Larvae were selected after the moult to third or fourth instar and divided into groups I, II and III as described above. Larvae from groups I and II were isolated individually in plastic cups to prevent cannibalism while 10 to 15 larvae from group III were kept per cup with artificial diet provided *ad libitum*. Larvae within each treatment were inoculated at the same time post-moult. Control larvae from groups I and II were kept without food for 12 to 15 h, then weighed and inoculated with SAHS; larvae from group III were fed for 12 to 15 h, then isolated as individuals, weighed, and kept without food for 2 h before inoculation. Treated larvae from groups I and II were kept without food for 14 to 17 h, then weighed and inoculated with Cry2A; larvae from group III were fed for 14 to 17 h, then isolated as individuals, weighed, and kept without food for 2 h before inoculation. Larvae were weighed every 24 h until pupation (moulting larvae were not weighed), and final pupal weight was recorded 24 h after the pupal moult. Larvae were reared at 27°C ± 2°C after inoculation.

Preliminary experiments were done to determine inoculation rate for Cry2A protein (data not shown). Larvae were inoculated *per os* (Keddie and Volkman, 1985) with 5 µl of Cry2A inclusion bodies containing 0.6 µg protein or SAHS (control). There were two replications; head capsules were retained and measured in the second replication. Head capsule width was determined by measuring the distance between the posterior left and

posterior right stemma. Measurements were made using a dissecting microscope and camera lucida with a Summasketch FX data tablet by Summagraphics.

### 4.3.3 Results

#### 4.3.3.1 Staining reactions in epithelial tissue wholemounts treated with B.t. toxins

DAPI uptake was confined to extruding columnar epithelial cells, damaged columnar cells and goblet cell cavities in untreated tissues or tissues treated with solubilization buffer as previously described (3.3.2) (data not shown).

A summary of staining results for B.t. protoxin treatments on epithelial tissue wholemounts is shown in Table 4.1. Staining reactions are reported only for columnar epithelial cells (col), extruding cells (ext) and small basal, presumably regenerative, cells (bas). Staining occurred in damaged columnar cells and was transitory in goblet cell cavities in all wholemount preparations as previously reported (3.3.2). Detailed staining reactions for each toxin on individual wholemounts are provided in Appendix 5. In most cases only data from the final observation for each preparation is presented. Numbers of stained cells are visual estimates, presented as values from 1 (less than 25% cells stained) to 4 (100% cells stained) (Appendix 5, Key). Blanks indicate lack of staining, not absence of cells. Overall tissue damage from dissection was recorded also (column 'td').

##### 4.3.3.1.1 Cry1Ac

DAPI staining of some individual wholemounts treated with Cry1Ac-LM are provided in Appendix 5.1.1 Staining results were as described previously (3.3.3). Treatment with Cry1Ac-LM resulted in DAPI uptake into large columnar epithelial cells, extruding cells and small basal cells in tissues dissected from moulting larvae. Stained

basal cells either were evenly distributed or in a regular array or circle around stained, large, fully differentiated columnar cells (see Figure 3.5). Tissues dissected from intermoult larvae had DAPI in medium- to large-sized fully differentiated columnar cells and extruding cells.

Cry1Ac-KH treatment of midgut epithelial tissue wholemounts from larvae moulting to fourth instar resulted in DAPI staining in fully differentiated columnar epithelial cells, small basal cells, and extruding columnar cells in all regions of the midgut. In tissue from one larva, all basal cells in the anterior region were stained by DAPI, and stained cells appeared in both nest-like (see Cry1Ab, below) and circular configurations (Appendix 5.1.2).

#### 4.3.3.1.2 Cry1Ab

Midgut epithelial tissue prepared from fourth instar larvae and treated with Cry1Ab protoxin had DAPI staining in columnar epithelial cells and extruding columnar cells in the anterior and middle regions of the midgut (Appendix 5.2). Fewer cells stained in the posterior region. In two wholemounts prepared 20 h post-moult, staining occurred in small basal cells (tissues observed 3⅓ h post-treatment).

In wholemounts prepared from insects dissected during the moult to either third or fourth instar and treated with Cry1Ab protoxin, DAPI staining varied with both protoxin concentration and time of wholemount production during the stadium (*i.e.* time of day tissue isolated). At the lowest concentration tested (0.55 µg protein) staining in all wholemounts occurred in many to all basal cells in all regions of the midgut. In some wholemounts some stained basal cells appeared in nest-like configurations (n, Appendix

5.2) (Figure 4.1). 'Nests' consisted of clusters of stained basal cells only; the regular distribution pattern of fully differentiated columnar epithelial cells was interrupted in these areas. Furthermore, in tissues with such a staining pattern usually there was little staining in neighbouring large columnar epithelial cells. Wholemounts which lacked this staining pattern had DAPI staining mainly in large columnar cells and small basal cells from the anterior and middle regions of the midgut. Few columnar cells were stained in the posterior region. In one wholemount stained basal cells appeared in a circular pattern (c) as described previously for wholemounts treated with Cry1Ac (3.3.3).

Most large columnar epithelial cells and small basal cells were stained by DAPI in tissues treated with 1.11 µg Cry1Ab protein. In the posterior region of two wholemounts only the basal cells present in 'nests' stained with DAPI while the majority of the basal regenerative cells remained unstained (observed up to 4½ h post-treatment). In the remaining wholemounts DAPI staining occurred in small basal cells which were scattered or in nest-like patterns (*i.e.* 4, n Appendix 5.2).

Staining occurred in most small basal cells and large columnar epithelial cells in all regions of the midgut at the highest Cry1Ab concentration tested (2.77 µg protein). Wholemounts produced earlier in the stadium had stained basal cells in both nest-like and circular patterns. These patterns of stained cells were not obvious in wholemounts produced later in the stadium.

#### 4.3.3.1.3 Cry1B

Midgut epithelial tissue prepared from third or fourth instar larvae and treated with Cry1B protoxin had DAPI staining in some medium-sized columnar epithelial cells and

extruding columnar cells (Appendix 5.3). Columnar cells were stained in all regions of the midgut but staining typically occurred in nuclei of cells along ridges. Stained basal cells appeared in a nest in two wholemounts.

In wholemounts prepared from insects dissected during the moult to either third or fourth instar and treated with Cry1B protoxin, staining occurred in small basal cells and extruding columnar epithelial cells. Stained basal cells appeared in nests in all regions of the midgut. In the posterior region these cells were not surrounded by stained columnar cells. Nuclear staining was confined to medium to large-sized columnar cells located on ridges in the anterior and middle regions.

#### 4.3.3.1.4 Cry1C

There was no staining in tissues prepared from a larva moulting to fourth instar when crude Cry1C-LM spore-crystal mixture was added, and staining occurred in some columnar epithelial cells from the middle region of the midgut after addition of a solubilized solution of this same mixture (Appendix 5.4.1).

Addition of solubilized lyophilized powder (Cry1C-KH) to tissues from third instar larvae resulted in variable staining of nuclei in columnar epithelial cells from all regions of the midgut (Appendix 5.4.2). Addition of protoxin to tissue prepared from a moulting larva resulted in DAPI uptake into a few large columnar epithelial cells. Small basal cells were unstained in tissues prepared from either moulting or intermoult larvae.

#### 4.3.3.1.5 Cry1D

DAPI staining in wholemounts prepared from third or fourth instar larvae occurred only in nuclei of columnar epithelial cells on ridges in the anterior and middle regions of

the midgut (Appendix 5.5). There was minimal staining in extruding cells in all regions of the midgut. Staining in scattered, small basal cells was apparent when tissues were viewed from the basal side. (When viewed from the apical side tightly packed unstained columnar cells obscured these cells.) In one wholemount stained basal cells appeared in a circular pattern surrounding unstained columnar cells.

In tissues from insects dissected during the moult to either third or fourth instar DAPI stained basal cells and only a few extruding columnar epithelial cells. In one wholemount stained basal cells were in a circular pattern.

#### 4.3.3.1.6 Cry1E

Staining in wholemounts prepared from third or fourth instar larvae and treated with Cry1E purified crystals was confined to some columnar epithelial cells on ridges or along tissue edges (r, e, Appendix 5.6). Only extruding columnar epithelial cells and damaged cells along cut edges were stained after tissues dissected from moulting larvae were given the same treatment.

Addition of solubilized Cry1E protoxin to tissue prepared from a fourth instar larva resulted in DAPI uptake into medium- to large-sized columnar epithelial cells in all regions of the midgut. Staining in tissues from larvae moulting to fourth instar occurred in the majority of basal regenerative cells and large columnar epithelial cells.

#### 4.3.3.1.7 Cry2A

Except for a few large and extruding columnar epithelial cells, tissues remained unstained after treatment with purified Cry2A inclusion bodies (Appendix 5.7).

Staining occurred in large and small-sized columnar epithelial cells and extruding

cells when solubilized Cry2A protoxin was added to tissue wholemounts prepared from larvae moulting to third instar larvae. Small basal cells were unstained.

#### 4.3.3.1.8 Cry3A

Epithelial tissue wholemounts treated with purified Cry3A protoxin remained unstained for up to 8 h post-treatment except for extruding columnar cells and damaged cells (including cells on ridges) (Appendix 5.8).

### 4.3.3.2 Insect bioassays

Bioassays were conducted here only to determine if toxins tested on epithelial wholemounts were toxic *in vivo*. Most assays were not replicated and numbers of insects assayed were low.

#### 4.3.3.2.1 Cry1Ab feeding bioassay

For Cry1Ab lyophilized powder, the $LD_{50}$ for *T. ni* was 109 ng protein if larvae were scored for mortality at day 14. Bacteria containing crystal inclusions were recovered from dead larvae treated with 200 and 332 ng protein (30% and 20% respectively).

#### 4.3.3.2.2 Cry1E feeding bioassay

The $LD_{50}$ for *T. ni* treated with Cry1E crystals was 6.3 ng protein if larvae were scored for mortality at day 14. *Bacillus*-type vegetative cells were recovered from some dead larvae treated with Cry1E concentrations of 100 ng or more.

#### 4.3.3.2.3 Cry1E oral inoculation bioassay

Mortality data for third and fourth instar *T. ni* larvae inoculated with 4 ng of Cry1E crystals is shown in Table 4.2. Data were not pooled because of starting weight

differences between instars. There were no differences between treatments for mean pupal weight for either instar even though mean starting weights were significantly different for group III third instar larvae and group I fourth instar larvae (P = 0.05; Student's *t*-Test). Days to pupation were significantly different between treatments for all groups of fourth instar larvae and for third instar larvae in groups I and II. Days to eclosion were significantly different between treatments for groups I and II for both instars. Low insect numbers and control mortality resulted in skewed corrected mortality values.

#### 4.3.3.2.4 Cry2A oral inoculation bioassay

Mortality data for fourth instar *T. ni* larvae inoculated with SAHS or 0.6 µg Cry2A inclusion bodies is shown in Table 4.3. Mortality for fourth instar larvae inoculated with Cry2A was highest in group III (52%, corrected for control mortality), and was significantly different than for insects inoculated with SAHS (P = 0.01, Chi-square test). Likewise, mortality for groups I and II were significantly different between treatments (P = 0.05 and 0.01 respectively; Chi-square test). There were significant differences between treatments for mean larval weight (3 d post-inoculation), number of days to pupation and number of days to eclosion (P = 0.01; Student's *t*-Test). Mean pupal weight for group III larvae treated with Cry2A was significantly different than for group III larvae treated with SAHS (P = 0.05; Student's *t*-Test).

All larvae selected for inoculation had just moulted to fourth instar. Mean starting weights were consistent within groups I and II; weights of larvae fed before inoculation were more variable. Under rearing conditions provided here, *T. ni* larvae have five larval

instars. Larvae from each group inoculated with Cry2A inclusions underwent a supernumerary moult, as did one insect from group I inoculated with SAHS (Table 4.4). Larvae moulted from fourth to fifth instar, then from fifth to sixth instar before the moult to pupa. Head capsules were saved after each insect moulted to confirm supernumerary moults and to measure head capsule widths (Table 4.4). Head capsules were retained also after pupal moults but their width was not measured (capsules split along the epicranial suture). There were no significant differences in head capsule width between treatments for the first moult (fourth to fifth instar) ($P = 0.05$; Student's *t*-Test).

## 4.4 Discussion

### 4.4.1 Midgut epithelial wholemounts treated with B.t. δ-endotoxins

DAPI staining occurred in columnar epithelial cells in *T. ni* wholemounts treated with B.t. protoxins (*i.e.* solubilized crystals) which are toxic to *T. ni in vivo*. As demonstrated earlier (Chapter 3) DAPI was excluded from untreated *T. ni* midgut columnar epithelial cells, and from cells treated with either solubilization buffer or *E. coli* HB101 culture products. This again confirmed that solubilized protoxin was converted to active toxin by midgut epithelial tissue. Staining of basal cells by protoxins known to have low *in vivo* toxicity to *T. ni* (Cry1B and Cry1D) indicated midgut epithelium also activated these protoxins. Lack of staining after addition of Cry3A protoxin was expected (since it is a coleopteran-specific toxin), but it was not determined if this was due to failure of *T. ni* epithelial wholemounts to activate Cry3A protoxin or due to lack of toxicity. (A simple experiment would answer that question *e.g.* addition of Cry3A toxin after its activation with beetle gut juice.)

In contrast, most columnar epithelial cells remained unstained by DAPI after treatment of wholemounts with non-solubilized crystals of any toxin. Addition of 650 ng Cry2A crystals resulted in little DAPI uptake in epithelial wholemounts, but addition of 590 ng crystals resulted in up to 52% mortality when inoculated *per os*. Likewise, addition of up to 600 ng of Cry1E crystals to epithelial wholemounts resulted in minimal DAPI uptake (staining occurred in some columnar cells on ridges or along edges in tissues from intermoult larvae treated with Cry1E crystals) whereas 50% mortality resulted from feeding 6.26 ng Cry1E crystals to *T. ni* in a diet surface contamination assay. DAPI staining was transitory in goblet cell cavities and in extruding columnar cells, but these reactions occurred in the absence of toxin (3.3.3). All columnar epithelial cells in one wholemount treated with Cry1Ac inclusions were permeable to DAPI (3.3.3.4) but in view of the results obtained with all other toxins that result may have been an anomaly. More experiments are necessary to determine if epithelial wholemounts can solubilize crystals, or if these results are indicative of levels of damage sustained during dissection.

With most toxins tested, DAPI staining reactions were consistent and stain intensity increased with time post-treatment. Inconsistent staining results in wholemounts after application of B.t. protoxins probably are due to several factors. They may indicate poor toxin distribution in fluid wells. Staining in wide areas adjacent to tissue edges (designated as 'e' in Appendix 5) when toxin concentrations were low likely indicated

insufficient toxin mixing[1]. This could be alleviated by pre-mixing protoxins with SAHS before delivery to the tissue. Because tissues are so fragile, mixing *in situ* is inadvisable. Also, PM formation may have interfered with access of stain or toxin to epithelial cells (see also 3.3.3.5). Irregular staining patterns observed after treatment of wholemounts with toxins at high concentration (*e.g.* Cry1C) likely indicated low protein toxicity. Alternately perhaps wholemounts were unable to fully activate Cry1C protoxin.

In wholemounts examined here, stained basal cells appeared in nests, in circular patterns, or evenly distributed over the basal surface (also 2.3.2, 3.3.3). Basal cell distribution patterns here parallelled regenerative cell patterns in *Manduca sexta*, where cell distribution changes over the moult period (Baldwin and Hakim, 1991). At pre-moult, *M. sexta* stem cells are scattered among differentiated epithelial cells. As midgut contents clear, stem cells divide; those clustered in groups are near goblet cells. (This preferred localisation disappears as stem cells proliferate.) Stem cells remain undifferentiated but their daughter cells increase in number until they outnumber differentiated cells and form a continuous basal layer intercalated with differentiated cells. Nuclear placement in undifferentiated daughter cells foretells eventual end products as cells differentiate (*i.e.* those with basal nuclei differentiate into goblet cells; those with apical nuclei become columnar cells). Differences in stained basal cell patterns in wholemounts produced here therefore likely reflect differences in regenerative cell distribution rather than differences in toxin affinity or binding. Patterns in stained

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[1] This was not the same as typical edge damage wherein one or two rows of cells along cut edges were damaged during dissection. That type of damage occurred even in the absence of toxin. Edge effects here after addition of protoxin were comprised of stained cells in patches or wide areas adjacent to cut edges but not extending across the centre of the tissue.

regenerative cells in intermoult larvae is consistent with the scenario presented for *M. sexta* if *T. ni* larvae were selected for dissection just preceding or following a moult.

Appearance of stained basal cells in nests was correlated with the time of day at which wholemounts were produced (*e.g.* Appendix 5.2): insects dissected later in the day following the moult did not exhibit the nest-like pattern of basal cell staining (most insects moulted between 18:00 and 21:00). Fully differentiated columnar epithelial cells were not present in these nests. In some wholemounts, even neighbouring columnar cells were unstained whereas cells in nests were stained by DAPI. With Cry1Ab this effect was seen only when low concentrations of toxin were applied: columnar cells surrounding nests were stained by DAPI when higher concentrations of Cry1Ab protoxin were added. Basal cells may be stained sooner than fully differentiated columnar cells simply due to their lower cell volume (*i.e.* easier access to the nucleus) and higher activity (more nucleic acids). With increased toxin concentration, DAPI staining in columnar cells would increase over time. Receptors on basal cells may have higher affinity for Cry1Ab toxin than receptors on neighbouring columnar cells. In a current model based on the structure of Cry3A, aggregates of six polymers bind to create a 20 Å pore (Yamamoto and Powell, 1993). If receptors on regenerative cells had higher affinity for Cry1Ab toxin, toxin molecules would bind there preferentially. A single bound molecule would then induce aggregation of enough molecules to create a pore, and pore formation would facilitate entry of DAPI. This aggregation phenomenon may differ among cell types and perhaps more molecules are required for pore formation in columnar cells. Alternately toxin binding and pore formation on regenerative cells may

differ from that on differentiated columnar cells. Perhaps toxin molecules bind to columnar cells without inserting into the membrane and creating pores. Knowles and Ellar (1986) report many cell lines are capable of binding B.t. toxin without showing cytopathic effects.

#### 4.4.1.1 Cry1Ac

Cry1Ac protoxin prepared from lyophilized spore-crystal powders was used here only to see if staining results were the same as for Cry1Ac produced in *E. coli* (3.3.3). DAPI staining after wholemount treatment with Cry1Ac from either source was the same: nuclei in columnar epithelial cells (including extruding cells and small basal cells) from all regions of the midgut stained blue (DAPI staining in goblet cell cavities was transitory). These results therefore verify earlier results using Cry1Ac from a different source (Cry1Ac-LM). Moar *et al.* (1990) also found that Cry1Ac toxicity for *T. ni* did not vary with the source of the isolate.

Stained basal cells were in nests, in circular patterns or randomly distributed. After treatment with Cry1Ac protoxin, staining typically occurred in every basal cell and every differentiated columnar cell in wholemounts from moulting insects. Tight packing of large columnar cells sometimes limited viewing of more basally-located cells (*e.g.* in wholemounts treated with Cry1Ac-KH, basal cell staining was best seen when tissues were observed from the basal surface).

#### 4.4.1.2 Cry1Ab

Staining occurred in most small basal cells and large columnar epithelial cells in all regions of the midgut in wholemounts produced from moulting insects. Staining in

columnar epithelial cells from the posterior region of the midgut increased with increasing protoxin concentration, which may indicate fewer receptors on cells in that region. Lack of staining in columnar cells in the posterior region in wholemounts prepared from larvae inoculated with Cry1Ac was reported earlier (3.3.1.3) even though addition of Cry1Ac directly to wholemounts from untreated larvae resulted in DAPI staining in columnar cells. In whole insects, Cry1Ab proteins may be retained in the anterior ⅔ of the midgut through reduced midgut motility or unequal distribution of receptors (Bravo *et al.*, 1992). Here perhaps unequal production or distribution of digestive enzymes prevented activation of toxin in the posterior region.

Overall staining after treatment with Cry1Ab was similar to that for Cry1Ac. Cry1Ac and Cry1Ab are very closely related: Cry1A toxins have an amino acid similarity of 82-90% (Höfte and Whiteley, 1989) and research has shown that they likely bind to the same receptor. Lee *et al.* (1995) agree with the model proposed by Van Rie *et al.* (1989) in which one population of binding sites (on receptor A) interacts with Cry1Aa, Cry1Ab, and Cry1Ac, receptor B binds Cry1Ab and Cry1Ac, and receptor C binds only Cry1Ac. Estada and Ferré (1994) determined that a line of *T. ni* selected with Cry1Ab developed resistance to Cry1Ab but not the closely related Cry1Aa or Cry1Ac (Cry1Ac and Cry1Ab compete for the same binding sites in *T. ni* BBMVs) so they concluded that Cry1Ab and Cry1Ac share the same high-affinity binding site, whereas Cry1Aa binds to a different receptor. They also report that Cry1Ac has a high level of non-specific binding, suggestive of a nonsaturable type of binding.

Several studies have compared toxicity of Cry1Ab and Cry1Ac to *T. ni*[2]. MacIntosh *et al.* (1990) report an $LC_{50}$ of 0.09 µg/ml for Cry1Ac and 0.19 µg/ml for Cry1Ab in a diet incorporation bioassay performed on neonate *T. ni* larvae. They observed no synergism when B.t. Cry1 proteins were combined. Estada and Ferré (1994) showed that *T. ni* neonates (mortality scored after 5 d) were susceptible to solubilized trypsin-treated Cry1Aa, Cry1Ab, and Cry1Ac but not to Cry1B or Cry1D ($LD_{50}$ values were 570, 480, 320, 5640, and 2530 ng/cm$^2$ respectively in a diet surface contamination assay). This toxicity correlated with toxin binding to BBMVs. Von Tersch *et al.* (1991) report an $LC_{50}$ of between 6.2 and 19 ng protein/cm$^2$ for neonate *T. ni* treated with Cry1Ac in a diet surface contamination bioassay. Moar *et al.* (1990) found that $LD_{50}$ values for Cry1Ac and Cry1Ab were not significantly different although Cry1Ac was slightly more toxic to *T. ni* neonates in a diet incorporation bioassay scored for mortality after 7 d ($LD_{50}$s of 0.10 - 0.31 µg/ml diet compared with 0.71 - 1.23 µg/ml diet). Here, the $LD_{50}$ for a diet surface contamination assay of lyophilized spore-crystal mixture was 109 ng protein when mortality was scored at adult maturity. (Protein content was determined for the spore-crystal mixture and likely was partly composed of cell components and spore proteins.)

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[2] Dulmage (1981) reports tremendous variation in activity and toxicity between B.t. strains. Dubois (cited in Dulmage *et al.*, 1981) classified potencies of isolates against *Lymantria dispar* as follows: very potent (> 80% mortality at 1.56 µg/ml; moderately potent (< 80% mortality at 1.56 µg/ml); weakly potent (< 80% mortality at 6.25 µg/ml); and nonpotent (< 80% mortality at 25 µg/ml). Faust and Bulla (1982) devised a scale of toxicity in *Bombyx mori* as follows: very high (< 0.25 µg/g); high (< 1.0 µg/g); moderate (1-3 µg/g); low (3-10 µg/g); and very low (> 10 µg/g).

#### 4.4.1.3 Cry1B

Reduced DAPI uptake in columnar cells after Cry1B treatment (even at 3.20 µg protein) indicated a weaker toxic reaction than that seen for Cry1Ab or Cry1Ac. Staining occurred in basal cells in tissues prepared from both moulting and intermoult larvae; staining in columnar cells was primarily in extruding cells and in cells along ridge tops. Staining of cells on ridge tops alone occurred in untreated tissues and is not indicative of a strong toxic reaction (3.4.3). Here, staining in wholemounts from intermoult larvae after Cry1B treatment was more than for tissues treated with SAHS but less than for tissues treated with Cry1Ac and Cry1Ab. *T. ni* completely empties its gut during the moult and does not resume feeding until moulting is concluded (Barbehenn and Keddie, 1992). Staining in basal cells alone therefore would not be indicative of a comparable toxic reaction *in vivo* since those cells normally would not be in contact with toxin in midgut lumen. Perhaps receptors for Cry1B on regenerative cells are altered when cells differentiate into columnar cells.

Cry1B has dual specificity for lepidopteran and coleopteran larvae (Bradley *et al.*, 1995). Although Höfte and Whiteley (1989) report 55-56% similarity between Cry1A proteins and Cry1B, Feitelson *et al.* (1992) and Yamamoto and Powell (1993) show that once activated, Cry1B toxin is closely related to activated Cry3 toxin. Specificity is due to differential processing of toxin in the insect's midgut: solubilized Cry1B is toxic to Colorado potato beetle, *Leptinotarsa decemlineata*; Cry1B does not require solubilization to be toxic to cottonwood leaf beetle, *Chrysomela scripta* or tobacco hornworm, *M. sexta*; and trypsin digestion of Cry1B lowers insecticidal activity of Cry1B against *M. sexta*

(Bradley *et al.*, 1995). The toxic domain of Cry1B for both Lepidoptera and Coleoptera is contained in a protein fragment of 65 kD (defined by amino acids 33 to 636) and is homologous to the toxic domains for other Cry1, Cry3 and Cry4 toxins, suggesting the mechanism for toxicity involves insertion and pore formation (Knowles and Ellar, 1987).

Based on results of wholemount staining here, *T. ni* is only weakly susceptible to Cry1B. Estada and Ferré (1994) show that *T. ni* neonates (mortality scored after 5 d) were more susceptible to solubilized trypsin-treated Cry1Aa, Cry1Ab, and Cry1Ac than to Cry1B or Cry1D (toxicity for Cry1B was 17 times less than for Cry1Ac; toxicity of Cry1D was about 8 times less). These toxicity data correlated to toxin binding to BBMVs.

#### 4.4.1.4 Cry1C

Variable staining in all regions of the midgut in epithelial tissues from third instar larvae at concentrations up to 3.98 μg protein indicated Cry1C is less toxic to *T. ni* than either Cry1Ab, Cry1Ac or Cry2A. Inconsistent staining patterns still occurred when high concentrations of toxin were added, with slightly more DAPI uptake in the posterior region of the midgut. This may signify non-specific binding or reduced toxicity.

Only two wholemounts from moulting larvae were treated with solubilized Cry1C, and in those there was very little staining after toxin treatment. This is different from the staining reaction seen with Cry1Ab, Cry1Ac or Cry2A, indicating a different mode of action or a different receptor complex. Within the lepidopteran-specific toxins, Cry1C shows a marked difference in insecticidal specificity compared to Cry1Aa (Van Rie *et al.*, 1989), a toxin very closely related to Cry1Ab and Cry1Ac (Höfte and Whiteley, 1989;

Feitelson *et al.*, 1992). In addition, Cry1C has only about 25% amino acid sequence homology with Cry2A in amino acid domains 1-640 (Feitelson *et al.*, 1992). In view of these reports it is not surprising that staining in wholemounts after treatment with Cry1C was different than that seen with Cry1Ab, Cry1Ac, or Cry2A.

#### 4.4.1.5 Cry1D

Toxicity of Cry1D for *T. ni* is considerably less than for Cry1Ac (Estada and Ferré, 1994) even though they share 70% amino acid sequence homology (Höfte and Whiteley, 1989). Here, DAPI staining in tissues from intermoult larvae was confined mainly to columnar cells on ridges and extruding columnar cells, which may be indicative of dissection damage or normal cell degeneration. All basal cells were permeable to DAPI after tissues from moulting insects were treated with Cry1D. Again, staining in basal cells would not correlate with toxicity *in vivo* since *T. ni* does not feed or have food present in the midgut during moulting. As with Cry1B, receptors present on regenerative cells were not expressed when cells were fully differentiated. Alternately, toxins were not able to bind to receptors present on differentiated cells or could not insert into membranes to form pores. Previous results (2.3.3, 3.3.2.) showed that staining in these basal cells was not due to solubilization buffer, dispase treatment or dissection technique. If receptors for B.t. toxins are endogenous digestive enzymes (*e.g.* aminopeptidase N) their primary role is not binding B.t. toxins. One can then speculate in this case that receptors on basal cells are different, have a different conformation or are altered before cells become fully differentiated so that they are no longer capable of binding Cry1D (or Cry1B) toxin.

#### 4.4.1.6 Cry1E

DAPI staining occurred in large columnar epithelial cells and small basal cells in tissues from moulting insects treated with solubilized Cry1E, and in small to medium sized cells from intermoult larvae. This staining pattern is similar to that after Cry1Ac treatment. Feitelson *et al.* (1992 ) show that these two toxins have more than 65% sequence homology.

Toxicity results from a feeding bioassay here confirm that Cry1E is highly toxic to *T. ni*: the $LD_{50}$ was 6.3 ng protein when mortality was scored at day 14 post-treatment (4.3.3.2.2). Results presented earlier (3.3.1.1) showed that the $LD_{50}$ for Cry1Ac crystals was 10 ng when mortality was scored at adult maturity. *Per os* inoculation of 4 ng resulted in increased larval duration and time to eclosion.

#### 4.4.1.7 Cry2A

DAPI staining occurred in large and small sized columnar epithelial cells after wholemounts were treated with solubilized Cry2A (protoxin). Staining did not occur in small basal cells in wholemounts prepared from moulting insects. This staining reaction was different from that seen with Cry1Ac-treated tissues, where both fully differentiated columnar cells and small basal cells were stained by DAPI. Dissimilar staining reactions of epithelial cells treated with Cry1Ac and Cry2A may indicate that Cry1Ac and Cry2A do not bind to the same receptor. The Cry2A protein is toxic to both Lepidoptera and Diptera (Höfte and Whiteley, 1989) but shares only 39% homology along a 100-amino acid segment with Cry1 proteins (Donovan *et al.*, 1988). Lack of staining in basal cells after treatment with Cry2A indicated that receptors for Cry2A were altered or not present

in regenerative cells, or toxins were not able to insert into the membrane at that specific time in the cell cycle. Obviously receptors were present later in the cell's life because large columnar cells did become stained by DAPI.

The consistent staining reaction of columnar cells in tissues treated with solubilized Cry2A is indicative of that protein's high toxicity towards *T. ni*. The $LC_{50}$ for *T. ni* neonates treated with P2 protein (now known as Cry2A) in a diet incorporation bioassay and scored for mortality at day 7 is 0.311 µg/ml (Yamamoto and McLaughlin, 1981). In a similar bioassay, Moar *et al.* (1994) report an $LC_{50}$ of 4.3 µg/g for Cry2A when mortality is scored at day 5. Results from the Cry2A inoculation bioassay here confirmed that toxicity (see 4.3.3.2.4).

#### 4.4.1.8 Cry3A

Epithelial cells (except extruding columnar cells and goblet cell cavities) in tissues prepared from either moulting or intermoult larvae and treated with solubilized Cry3A protoxin remained unstained by DAPI. Even small basal cells were unaffected. This experiment validates the midgut assay as a model for evaluation of B.t. toxicity. Based on host range, Cry3A is a coleopteran-specific toxin (Höfte and Whiteley, 1989) so addition of Cry3A protoxin to wholemounts should not result in DAPI uptake. Here, uptake signifies an alteration in epithelial cell membrane permeability in tissues and is caused by treatment with B.t. toxins to which the insect is sensitive. MacIntosh *et al.* (1990) report *T. ni* neonates are insensitive to 500 µg/ml Cry3A in a diet incorporation bioassay.

### 4.4.2 Insect bioassays

All feeding or inoculation bioassays conducted here confirmed that the B.t. toxins used in those assays were toxic to *T. ni*. Addition of Cry2A or Cry1E crystals resulted in little DAPI uptake in epithelial wholemounts but did cause mortality when inoculated into or fed to *T. ni*. Cry1Ab also was toxic to *T. ni*: addition of 109 ng lyophilized spore-crystal powder resulted in 50% mortality in a diet-incorporation bioassay.

#### 4.4.2.1 Cry1E oral inoculation bioassay

Significant differences between treatments for days to pupation and eclosion coupled with no differences in pupal weight and low mortality indicated Cry1E inoculation caused increased larval duration with possible teratogenic effects. Since spores were present in the inoculum (hence recovery of *Bacillus* from necropsies in Cry1E feeding bioassay) it was possible they contributed to these effects. Ramachandran *et al.* (1993) found that although pupal weights were not affected, larval duration increased after *Choristoneura fumiferana* were exposed to Cry1Aa crystal inclusions. Insect numbers were too low and control mortality was too high to draw conclusions from this test however it was noted that most larvae inoculated with Cry1E died as pupae, indicating possible teratogenic effects.

#### 4.4.2.2 Cry2A oral inoculation bioassay

Although mean larval weights taken 3 d post-inoculation with Cry2A inclusions were significantly different between larvae inoculated with SAHS and Cry2A inclusions, the only significant difference in mean pupal weight was in larvae fed before inoculation with Cry2A. Larvae inoculated with Cry2A did take longer to pupate and eclose, and

were apparently able to regain lost weight before pupation. This may have had something to do with the extra moult cycle. It is interesting to note that Cry2A was the only toxin tested here to cause induction of supernumerary moults in inoculation bioassays. Initiation of supernumerary moults may signify a mechanism to overcome toxic effects of Cry2A. If insects respond to a single sublethal dose of Cry2A by immediately beginning another moult cycle, affected columnar cells would slough off and be replaced by differentiation of unaffected basal cells. Retnakaran *et al.* (1983) describe a similar situation in *C. fumiferana* in which a sublethal dose of B.t. results in feeding inhibition followed by midgut repair through replacement of destroyed cells by differentiation of unaffected cells from regenerative nidi. When inoculation levels are high, presumably the rate of cell destruction is greater than the rate at which cells are replaced by nidi, resulting in disruption of midgut ion regulation and death. Another feature that distinguishes Cry2A from the other toxins tested here is that it did not cause DAPI uptake into basal cells along with columnar cells when added to epithelial tissue wholemounts. Conclusions cannot be drawn based on only four wholemounts from moulting insects treated with solubilized Cry2A, but this is an area worth future research. Although only two wholemounts from moulting larvae were examined, addition of Cry1C gave similar results: basal cells remained unstained by DAPI. The corresponding inoculation bioassay was not done so it is unknown if induction of supernumerary moults was correlated with this lack of basal staining. Certainly other toxins did not produce similar results in bioassays (Cry1Ac feeding bioassay, 3.3.1.1, and Cry1E and Cry1Ab bioassays), and staining in wholemounts treated with those toxins resulted in DAPI

uptake into basal cells.

## 4.5 Conclusions

Addition of various B.t. toxins to midgut epithelial tissue wholemounts has demonstrated the usefulness of this midgut model as an assay method. Different B.t. toxins produce different patterns of DAPI staining in epithelial cells. First, epithelial wholemounts were capable of converting solubilized toxins (protoxins) to activated toxins (as demonstrated by DAPI uptake in columnar and basal cells in epithelial wholemounts). Second, DAPI uptake after addition of Cry1Ac from independent sources produced similar staining patterns: all columnar and basal cells became permeable to DAPI. Third, Cry3A, a toxin specific to Coleoptera, had no effect on DAPI uptake into columnar epithelial cells. From this I concluded that pores were not formed and columnar cell membrane permeability was not affected. Fourth, staining reactions among toxins with highest degree of amino acid homology *(i.e.* Cry1Ac and Cry1Ab; greater than 86% sequence homology) (Höfte and Whiteley, 1989) were similar whereas staining after addition of a dissimilar toxin (Cry2A) produced different results. And fifth, there was only minimal DAPI staining after addition of toxins with low *in vivo* toxicity (Cry1B and Cry1D).

If staining patterns are characteristic of each toxin or class of closely related toxins, it may be possible to partially identify which genes may be present in new isolates. This obviously would work only if new isolates had genes for only one toxin. If isolates had combinations of dissimilar genes which affected permeability in different cells, these effects would be additive (*i.e.* it would be impossible to identify which gene was

responsible for toxicity if all cells were stained by DAPI). The greatest value of this midgut assay system would be the identification of new isolates toxic to *T. ni*. Here, there was good correlation between toxicity *in vivo* (bioassays here and in the literature) and DAPI uptake into susceptible midgut columnar epithelial cells. This assay should be useful in monitoring development of resistance in the field or identifying changes in cell permeability in laboratory-induced resistance. Once adapted for other insects it also would provide a quick means of determining effects of B.t. spray programs on non-target insects.

Figure 4.1 Epithelial wholemount prepared from moulting *T. ni* larva treated with Cry1Ab protoxin and stained with DAPI. Note stained basal cells in nests (N) (U1 filter). Insert: nest surrounded by columnar cells (3 x enlargement) (DYLT filter). Scale bar = 25 μm.

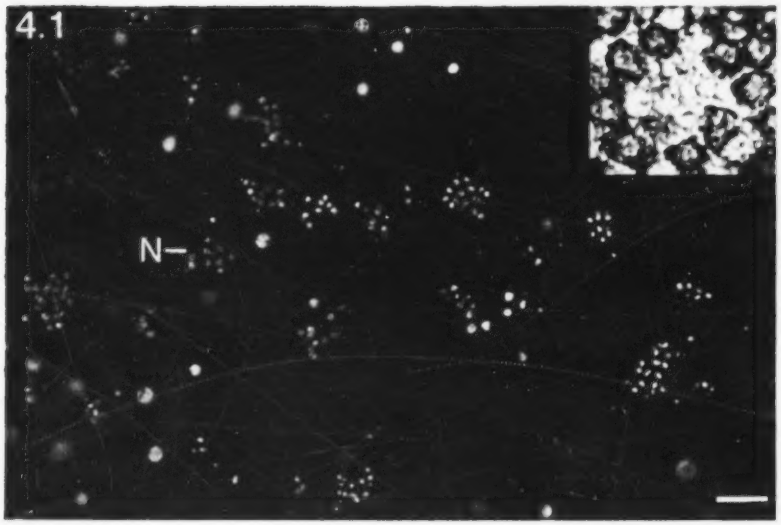

Table 4.1 DAPI uptake in epithelium tissue wholemounts prepared from moulting or intermoult *T. ni* larvae after treatment with B.t. protoxins. (Staining reactions in goblet cells and damaged cells not included.)

| Protoxin | Moulting | | | Intermoult | | |
|---|---|---|---|---|---|---|
| | Cell Type | | | Cell Type | | |
| | col | ext | bas | col | ext | bas |
| Cry1Ac | + | + | + | + | + | + |
| Cry1Ab | + | + | + | + | + | + |
| Cry1B | r | + | + | r | + | + |
| Cry1C | + | - | - | + | + | - |
| Cry1D | r | + | + | r | + | + |
| Cry1E | + | + | + | + | - | - |
| Cry2A | + | + | - | nd | nd | nd |
| Cry3A | - | + | - | r | + | - |

| | |
|---|---|
| col | columnar epithelial cells |
| ext | extruding columnar epithelial cells |
| bas | small basal cells |
| + | stain uptake into cell |
| - | no stain uptake into cell |
| c | stained cells in circular pattern |
| n | stained cells in nest-like pattern |
| r | stained nuclei in cells on ridges |
| nd | not done |

Table 4.2. Comparison of third and fourth instar *T. ni* larvae inoculated with SAHS or 4 ng Cry1E crystals.

| **Third instar** Treatment | n | Mean Larval Weight (mg) Start (mean ± S.D.) | n | Mean Pupal Weight (mg) (mean ± S.D.) | No. Days to Pupation (mean ± S.D.) | No. Days to Eclosion (mean ± S.D.) | n | Mortality | Abbott's Corrected Mortality |
|---|---|---|---|---|---|---|---|---|---|
| SAHS - Group I | 8 | 5.6 ± 0.9 | 8 | 250.0 ± 17.0 | 8.0 ± 0.0 | 13.6 ± 0.5 | 0 | 0% | |
| SAHS - Group II | 12 | 5.8 ± 1.1 | 10 | 248.0 ± 31.6 | 8.1 ± 0.3 | 13.4 ± 0.5 | 4 | 33% | |
| SAHS - Group III | 2 | 21.8 ± 0.0 | 1 | 266.2 | 7.0 | 13.0 | 1 | 50% | |
| Cry1E - Group I | 8 | 5.1 ± 1.4 | 8 | 255.3 ± 15.7 | 8.9 ± 0.4** | 14.3 ± 0.5* | 1 | 13% | 13% |
| Cry1E - Group II | 12 | 6.7 ± 1.7 | 10 | 245.8 ± 30.9 | 8.9 ± 0.6** | 14.6 ± 0.7** | 4 | 33% | 0% |
| Cry1E - Group III | 4 | 15.3 ± 2.2* | 3 | 253.9 ± 14.3 | 7.7 ± 1.2 | 13.0 ± 1.7 | 1 | 25% | -50% |

| **Fourth instar** Treatment | n | Mean Larval Weight (mg) Start (mean ± S.D.) | n | Mean Pupal Weight (mg) (mean ± S.D.) | No. Days to Pupation (mean ± S.D.) | No. Days to Eclosion (mean ± S.D.) | n | Mortality | Abbott's Corrected Mortality |
|---|---|---|---|---|---|---|---|---|---|
| SAHS - Group I | 7 | 9.2 ± 1.0 | 7 | 225.3 ± 17.4 | 7.0 ± 0.0 | 12.2 ± 0.4 | 1 | 14% | |
| SAHS - Group II | 3 | 9.9 ± 1.0 | 2 | 236.4 ± 4.2 | 7.0 ± 0.0 | 12.0 ± 0.0 | 1 | 33% | |
| SAHS - Group III | 13 | 22.7 ± 5.4 | 12 | 234.7 ± 13.9 | 6.7 ± 0.7 | 12.2 ± 0.6 | 1 | 8% | |
| Cry1E - Group I | 11 | 12.7 ± 4.1* | 9 | 235.3 ± 31.8 | 7.8 ± 1.0* | 13.1 ± 1.4* | 2 | 18% | 5% |
| Cry1E - Group II | 11 | 9.9 ± 1.5 | 10 | 240.2 ± 20.9 | 7.5 ± 0.7* | 12.8 ± 1.1* | 1 | 9% | -36% |
| Cry1E - Group III | 15 | 22.8 ± 5.6 | 11 | 227.4 ± 25.6 | 7.2 ± 0.8* | 12.2 ± 0.6 | 5 | 33% | 28% |

Group I unfed; did not eat cast exuvium or diet
Group II unfed; ate cast exuvium only
Group III fed; ate artificial diet (± cast exuvium)
SAHS sterile artificial haemolymph solution
moulting larvae were not weighed
* denotes significance at P=0.05; Student's t test.
** denotes significance at P=0.01; Student's t test.

Larvae were reared at 27°C ± 2°C

Table 4.3 Comparison of fourth instar *T. ni* larvae inoculated with SAHS or 0.59 µg Cry2A inclusion bodies.

| Treatment | n | Mean Larval Weight (mg) Start (mean ± S.D.) | n | after 3 d (mean ± S.D.) | n | Mean Pupal Weight (mg) (mean ± S.D.) | No. Days to Pupation (mean ± S.D.) | No. Days to Eclosion (mean ± S.D.) | n | Mortality | Abbott's Corrected Mortality |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SAHS - Group I | 39 | 9.4 ± 1.1 | 25 | 167.6 ± 21.3 | 33 | 221.1 ± 19.9 | 7.1 ± 0.5 | 12.1 ± 0.5 | 7 | 18% | |
| SAHS - Group II | 39 | 9.8 ± 1.4 | 25 | 173.2 ± 18.9 | 33 | 235.1 ± 17.2 | 7.2 ± 0.5 | 12.2 ± 0.5 | 5 | 13% | |
| SAHS - Group III | 40 | 33.9 ± 7.2 | 27 | 184.7 ± 72.1 | 30 | 237.5 ± 20.7 | 6.0 ± 0.4 | 11.1 ± 0.4 | 11 | 28% | |
| Cry1Ac - Group I | 69 | 9.3 ± 1.2 | 29 | 58.7 ± 22.3** | 44 | 231.2 ± 36.3ns | 9.3 ± 0.8** | 14.3 ± 1.00** | 29 | 42% | 29%* |
| Cry1Ac - Group II | 58 | 9.3 ± 1.4 | 31 | 52.7 ± 18.2** | 38 | 230.4 ± 27.5ns | 9.7 ± 0.8** | 14.8 ± 1.1** | 24 | 41% | 33%** |
| Cry1Ac - Group III | 66 | 24.6 ± 5.7 | 18 | 70.6 ±32.2** | 26 | 222.8 ± 32.5* | 8.3 ± 0.9** | 13.3 ± 0.9** | 43 | 65% | 52%** |

Group I unfed; did not eat cast exuvium or iet
Group II unfed; ate cast exuvium only
Group III fed; ate artificial diet (± cast exuvium)
SAHS sterile artificial haemolymph solution
moulting larvae were not weighed
** denotes significance at 0.01
* denotes significance at 0.5
ns not significant

Larvae were reared at 27°C ± 2°C

Table 4.4 Numbers of *T. ni* larvae inoculated with Cry2A inclusions which underwent supernumerary moults and head capsule widths for larvae moulting from fourth to fifth and from fifth to sixth instar.

| | | Supernumerary moult | | Head capsule width (mm) (mean ± SD) | |
|---|---|---|---|---|---|
| Treatment | n | n | % | 4 to 5 | 5 to 6 |
| SAHS - Group I | 39 | 1 | 3% | 1.18±0.03 | |
| SAHS - Group II | 39 | 0 | 0% | 1.19±0.03 | |
| SAHS - Group III | 40 | 0 | 0% | 1.16±0.03 | |
| Cry1Ac - Group I | 69 | 50 | 72% | 1.19±0.03 | 1.51±0.12 |
| Cry1Ac - Group II | 58 | 46 | 79% | 1.21±0.09 | 1.50±0.13 |
| Cry1Ac - Group III | 66 | 22 | 33% | 1.13±0.06 | 1.55±0.02 |

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## Chapter 5 Into the future with *Bacillus thuringiensis* δ-endotoxins

### 5.1 Risk assessment of field applications/transgenic plants

The greatest concerns in the field of microbiological control of insects are not related to discovery of control agents or their efficacy, but rather risk factors associated with release of genetically engineered organisms and development of insect resistance to naturally occurring biological control agents. Products containing some form of *Bacillus thuringiensis* (B.t.) constitute 80 - 90% of the microbial pesticides purchased and used (Levin, 1995). *Bacillus thuringiensis berliner* was registered for use in 1961. Since then, different mixtures of naturally produced species or toxins have been registered. Genetic manipulation of these natural strains has taken advantage of natural forms of genetic recombination including conjugation, transduction, and transformation, which result in expression of exchanged information. Since these processes all occur in nature the modified organisms have not been of great concern to regulatory agencies. Genetically engineered microorganisms are produced when foreign DNA is inserted into natural microorganisms, and these are the organisms which require additional regulatory scrutiny.

Risk assessment involves detailed knowledge of parent and transgenic organisms, methods and frequency of introduction, toxicity to target and non-target organisms as well as site characteristics and ecological effects (Tzotzos, 1995). B.t. is in hazard group one, those organisms that are most unlikely to cause human disease. Nevertheless broader environmental concerns must be addressed. "Ecological effects" is last on the Indicative List of "Data Requirements for Biological Risk Assessment" (Tzotzos, 1995).

So far there is very little known on the natural role of B.t. in the environment. Overuse of B.t. in spray programs may upset a delicate natural biological control system or an intrinsic component of soil ecology. Indiscriminant broadcasting of B.t. in the environment would likely increase insect-bacteria encounters and could result in death of beneficial or non-target insects. Some B.t. strains have been found which are toxic to protozoa, organisms which play a large role in regulation of bacteria in aquatic and soil systems. Proteins produced by cry5 and cry6 genes are active against nematodes (Feitelson *et al.*, 1992), important detritovores and carnivores found in virtually every habitat on earth. Until effects of B.t. spray programs are known on these important soil and aquatic organisms little can be deduced on environmental impacts.

Most B.t. genes coding for insecticidal proteins are located on plasmids and *Bacillus* species have constitutive competence for transformation (Höfte and Whiteley, 1989; Lorenz and Wackernagel, 1994). Broad host-range plasmids and transposons pass between all major branches of the prokaryotic kingdom (Lorenz and Wackernagel, 1994). Plasmids have been identified that will transfer DNA between gram-positive and gram-negative bacteria and between a bacterium and yeast (Fincham and Ravetz, 1991). Purified plasmid DNA can remain intact in soil for two to five days before degradation begins, and DNA molecules may persist in soil for weeks or even months (Lorenz and Wackernagel, 1994). Habitats which favour high bacterial contamination (*e.g.* insect gut) favour transformation by free DNA (Lorenz and Wackernagel, 1994). This provides possibilities for development of novel B.t. toxins through genetic exchange and recombination events, but long-term consequences are unknown. The only possible way

to limit introduction of DNA into the environment is to limit introductions of bacteria containing toxins or ensure the donor containing the insecticidal crystal protein (ICP) is dead. One such delivery system has been developed by Mycogen in which B.t. toxin genes are cloned into *Pseudomonas fluorescens* cells which are killed after fermentation (Feitelson *et al.*, 1992).

Another important consideration is the incorporation of genetic markers, such as antibiotic resistance, which will be released into the environment along with the genetically modified organism (GMO). Resistance to penicillin, ampicillin, tetracycline and chloramphenicol already occur widely in the environment, so it is thought their release in GMOs would not cause a problem (Grinstead, 1995). Yet widespread release of plasmids containing antibiotic resistance can only add to what has been described as a worldwide crisis of antibiotic resistance (Neu, 1992).

### 5.2 Development of insect resistance to B.t.

Recent concerns on environmental impacts of synthetic chemicals has lead to increased use of microbial control agents in both forestry and agriculture. Public pressure has lead to use of B.t. on Canadian crown-owned forestry tracts for control of insect pests *e.g. Bacillus thuringiensis* var. *kurstaki*, the main agent for biological control of spruce budworm, *Choristoneura fumiferana*, in Canada (van Frankenhuyzen and Payne, 1993). To date, no resistance has been demonstrated, however inconsistent efficacy has been correlated with lack of persistence of B.t. in the environment (van Frankenhuyzen, 1993). Improvements have been made in suspension media and delivery systems to overcome this problem (Feitelson *et al.* 1992; Payne and van Frankenhuyzen,

1995). The gene for Cry1Aa has been successfully introduced into white spruce, *Picea glauca*, using particle acceleration (Ellis *et al.*, 1993) and low levels of expression resulted in reduced larval weights in *C. fumiferana* (after 6 days exposure). But because several hundreds of generations of insects could occur in one generation of transgenic trees, biotype formation would likely accelerate resistance development (Raffa, 1989).

Use of B.t. on agricultural crops has increased substantially, with global sales estimated at $100 million annually in 1991 (MacDonald, 1991). Insect pest control on cotton in the USA has relied mainly on B.t. For example, cotton is a heavily managed crop in the US, requiring over $354 million per year in pesticides alone. In spite of high inputs, arthropod pests cause $273 million loss in fibre production (John and Stewart, 1992). Overuse of chemical insecticides has resulted in development of insect resistance and loss of natural predators. B.t. has been used to control insect pests on cotton for more than 30 years, and it is one biological agent selected for use in a genetic engineering strategy to produce transgenic plants. Lepidoptera are the most serious pests, including *Heliothis zea* (cotton bollworm), *Heliothis virescens* (tobacco budworm), and *Pectinophora gossypiella* (pink bollworm). Single gene proteins for Cry1Ab and Cry1Ac expressed in cotton provided effective protection against *H. zea* (Perlak *et al.*, 1990). Yet development of resistance to a single 130 kDa B.t. protein in laboratory selected *Heliothis* highlights future problems in use of single gene constructs in transgenic plants (Stone *et al.*, 1989). Since traditional preparations are spore-crystal combinations which contain the complete arsenal of B.t. toxins, development of resistance has been slow. This parallels use of multifunctional pesticides, such as arsenical insecticides and copper

fungicides, which have produced few cases of resistance (Georghiou, 1988). Transgenic plants containing single toxin genes may accelerate resistance development and one of the greatest natural insect control weapons available to date may be squandered.

The first generation of transgenic plants containing B.t. toxin genes provided high levels of δ-endotoxin in all tissues (Gould, 1988). By using transgenic seeds, farmers are providing control before pests are known, and selection pressures may lead to adapted insects that are not controlled by B.t. in the future. If primary pests are eliminated by B.t. and non-pest insects with marginal sensitivity are raised to pest status, control by further applications of B.t. would be ineffective. Georghiou (1988) suggests also that insect species with only marginal sensitivity towards B.t. would be more prone to resistance development than more sensitive species. Many insects display avoidance behaviour to B.t. and they may seek out alternate food sources in neighbouring crops. Nontarget insects might also be killed by transgenic plants. Conversely a big advantage for B.t. use is preservation of natural enemies which would not be targeted because of B.t. toxin specificity. Second generation B.t.-incorporated transgenic plants would be designed to limit toxin expression to specific plant parts (*i.e.* young cotton bolls only) or at specific times (*i.e.* induced by feeding damage). Improved technology will enable insertion of multiple or distantly related genes thereby delaying resistance development.

### 5.3 Problems encountered in B.t. research

Studies which compare whole insect toxicity bioassays using first instars with BBMV binding assays from last instars are common (Van Rie *et al.*, 1989; Garczynski *et al.*, 1991; Gould *et al.*, 1992) even though results from binding studies using BBMVs do

not always correlate with toxicity data in live insects (Gould *et al.*, 1992). Both Lepidoptera and Coleoptera have variable distribution of ICP binding sites in the midgut (Bravo *et al.*, 1992). Results presented here showed that epithelial cell populations change within an instar; so likely do receptor populations change within and between instars.

Differences in receptor binding affinity also lead to differences in toxicity in insects (*e.g.* Cry1Ab and Cry1Ac bind to the same receptor in *Ostrinia nubilalis* but Cry1Ac has lowered affinity which results in a 10-fold lower toxicity) (Denolf *et al.*, 1993). Cry1B does not share this receptor, but uses a second type of receptor, consistent with Hofmann *et al.*, (1988) and Van Rie *et al.*, (1989, 1990) who say there is a distinct family of receptors to which different toxins bind. One such receptor identified for Cry1Ac is aminopeptidase N, which is anchored in the membrane by a glycosyl-phosphatidyl inositol (GPI) linkage (Garczynski and Adang, 1995; Knight *et al.*, 1995). If receptors are endogenous midgut proteins present on columnar epithelial cells then lack of correlation with *in vitro* and *in vivo* results is hardly surprising. The two current models used to evaluate B.t. toxicity employ non-midgut cells (*i.e.* most cell lines) and disrupted membranes of midgut cells (BBMVs). Neither guarantee use of columnar epithelial cells or their constituents. Use of non-midgut derived insect cell lines probably contributes to differences in susceptibility between whole insects and cultured cells derived from them (Davidson, 1989; Johnson, 1994). B.t. toxins cause pore formation in planar lipid membranes (Schwartz *et al.*, 1993) which lack specific membrane receptor proteins, so there must be non-specific binding as well as a receptor-mediated response. Estada and

Ferré (1994) show that Cry1Ac has a high level of non-specific binding, suggestive of a nonsaturable type of binding.

## 5.4 Evaluation of new midgut epithelial tissue model[1]

By current standards for assessing tissue and cell viability (trypan blue exclusion), this epithelium tissue model provides living tissue for up to 24 h post-dissection. Here, the fluorochromes propidium iodide (PI) and DAPI (4',6-diamidino-2-phenylindole) behaved similarly: stains were excluded from untreated columnar epithelial cells and entered goblet cell cavities only transitorily. Viability as demonstrated by membrane exclusion does not ensure full physiological activity. In tissue produced here, production of peritrophic membrane (PM) and basal lamina and activation of protoxin to toxin demonstrated that epithelial wholemounts retained some normal physiological function.

Several controls were performed to confirm cell viability. Enzymatic removal of basal lamina and connective tissue from midgut epithelium did not result in loss of tissue viability or increased cell staining by trypan blue. Short exposure to UV radiation over several hours did not increase membrane permeability to the fluorochromes used. Likewise, addition of solubilization buffer and *E. coli* cell products did not result in changes in cell membrane permeability. Tissues treated with SAHS alone and stained with PI or DAPI resulted in staining in a sub-population of columnar epithelial cells, those which extruded above the rest of the tissue. These cells were in the normal process of degeneration.

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[1] Chapters two and three have been combined and submitted for publication under the title 'A new tissue technique for evaluating effects of *Bacillus thuringiensis* toxins on insect midgut epithelium'.

Apoptosis is the programmed death of cells destined for elimination during normal development. Apoptotic cells initially show exclusion of non-permeant dyes such as PI, whereas later stages of apoptosis are accompanied by an increase in membrane permeability, allowing PI to enter cells (Sun *et al.*, 1992). DAPI and PI also entered and exited goblet cell cavities. This staining had not been observed before, and probably occurred here because basal lamina and connective tissues were removed from the basal surface of the cells, allowing access to basally-located pumps. Stain which entered goblet cells was quickly pumped into cavities and released apically; cell cytoplasm and nuclei remained unstained. DAPI staining of small basally-located cells occurred after addition of some B.t. protoxins; it did not occur in tissues bathed in SAHS alone or in response to Cry1C, Cry2A or Cry3A. This indicated that basal cells (thought to be regenerative) may play some part in toxic response to B.t. protoxins. This type of response may partially explain why some insects are more susceptible to B.t. toxins. For example, if intoxicated larvae retain food in their guts during moulting, toxin will still be present. If presence of toxin results in columnar cell degeneration and sloughing, toxins may gain access to basal cells which are normally not in contact with midgut lumen. Death of basal regenerative cells would then remove any possibility of recovery in midgut epithelium.

Addition of solubilized B.t. protoxins affected membrane permeability in columnar epithelial cells, allowing entry of PI and DAPI. Therefore this tissue model was useful as an assay method to detect which B.t. proteins were toxic to *T. ni*. B.t. toxins are classified according to their host specificity (Cry 1 - Lepidoptera; Cry 2 - Diptera *etc.*)

and their amino acid sequences (Crickmore *et al.*, 1995). Closely related toxins may cause similar reactions in a host insect, both *in vivo* and at the cellular level. Here, closely related toxins Cry1Ac, Cry1Ab, and Cry1E had similar effects on *T. ni* midgut epithelial cells *in vitro*: DAPI stained most columnar epithelial cells and basal regenerative cells. These toxins were all toxic to *T. ni* in diet surface contamination bioassays.

Here, DAPI staining of columnar cells correlated with toxicity *in vivo*: DAPI staining did not occur after treatment of wholemounts with toxins lacking toxicity to *T. ni* (Cry3A), and toxins with reported low toxicity to *T. ni in vivo* (Cry1B and Cry1D) caused minimal changes in columnar cell membrane permeability in wholemounts. This is not always the case in studies using BBMVs. Cry1Ac shows saturable binding *in vitro* without showing toxicity *in vivo* to *Spodoptera frugiperda* (Garczynski *et* al., 1991), *Lymantria dispar* (Wolfersberger, 1990), and *Heliothis virescens* (Lee *et al.*, 1995). This further demonstrates the suitability of this midgut assay and illustrates its advantage over other midgut models.

Staining results from epithelial tissues prepared from whole insects inoculated with solubilization buffer or Cry1Ac protoxin were consistent with staining observed in wholemounts treated with SAHS and solubilization buffer: DAPI staining did not occur in columnar epithelial cells (other than extruding cells) in any wholemounts prepared from insects inoculated with solubilization buffer, but did occur in damaged cells along cut edges and transitorily in goblet cell cavities. In wholemounts prepared from larvae treated with Cry1Ac protoxin, columnar epithelial cells stained but only when assayed for

a limited time after inoculation, implying that cells in whole insects were able to recover from sub-lethal doses of Cry1Ac or were sloughed off.

I was not able to demonstrate cell recovery after treatment with B.t. protoxins in this model although some tissue recovery occurred through elimination of affected cells. Nishiitsutsuji-Uwo *et al.* (1979) demonstrate that by replacing medium containing activated toxin with fresh uncontaminated medium, swollen TN-368 cells recovered. They conclude that effects of δ-endotoxin are reversible if caught in time. However TN-368 cells are derived from adult *T. ni* ovaries and toxicity results probably do not parallel reactions in columnar epithelial cells. Since B.t. toxins cause pore formation even in artificial lipid bilayer membranes it is reasonable to assume they will do so in cells from susceptible insects. Here, addition of DAPI 15 h post-treatment with Cry1Ac protoxin and subsequent SAHS rinse resulted in staining in columnar cells, indicating pores remained open. Van Rie *et al.* (1989) report the degree of reversibility of binding may be correlated with the ability of other ligands to compete with the same binding site, and that partial irreversibility of binding seems a general feature of lepidopteran-specific ICPs. Regardless, once the toxin inserts into the membrane to form a pore, binding is irreversible. Lee *et al.* (1995) report Cry1Ac toxin binding is not reversible in either resistant or susceptible insects. It would therefore not be possible to demonstrate cell recovery in this assay. DAPI enters cells with altered membrane permeability and binds irreversibly to nuclear DNA. Cells thus altered remained stained by DAPI even if pores or channels later close.

Different staining reactions among toxins in epithelial tissues implies different cell

receptors, different epitopes on the same receptor, or different binding mechanisms. Additionally, receptors necessary for toxicity may be present, but there may be a difference in pore-forming ability of various toxins, or some receptors may be non-functional. Lee *et al.* (1995) suggest nonfunctional receptors may fail to promote integration of the toxin into the cell membrane, may fail in pore formation, or may have inhibition of toxin oligomerization.

Although columnar cells retain their ability to exclude dead-cell indicators, there is no proof that goblet cells retain their capacity to regulate ion movement and pH. In preliminary experiments pH measurements taken before and after addition of solubilization buffer (pH 9.5) or Cry1Ac protoxin to wholemounts indicated some micro-environmental regulation of pH, and this would likely result from ion regulation by goblet cells (Dow, 1992). Further evidence of goblet cell function is their ability to pump vital dyes out of cell cavities through the apical membrane. Lack of staining in goblet cell cytoplasm and nuclei either indicated goblet cells were completely empty or they remained alive and continued to pump. Presumably, if cells were dead, PI and DAPI would accumulate in the cells. Dow (1992) found that acridine orange was excluded from actively pumping goblet cells. Observations here supported this if allowances were made for the initial appearance of AO in cells before it was pumped out. SAHS provides more nutrients than the osmotically-balanced salt solutions used with midgut perfusion chambers, so perhaps goblet cells remain viable for longer than cells in that apparatus.

## 5.5 Future research

### 5.5.1 Midgut epithelial wholemounts from other insects

*Trichoplusia ni* was used here to develop a new model to study toxic effects of B.t. in Lepidoptera. Although data was not presented here, epithelial wholemounts were produced also in *Heliothis virescens* (tobacco budworm) and *Spodoptera frugiperda* (fall armyworm) (at Ciba-Geigy Corporation, Research Triangle Park, North Carolina), *Manduca sexta* (tobacco hornworm) *Mamestra brassicae* (Bertha armyworm), and *Leptinotarsa decemlineata* (Colorado potato beetle). Initial attempts on the migratory grasshopper, *Melanoplus sanguinipes* and forest tent caterpillar, *Malacosoma disstria*, failed. Staining results on wholemounts from *H. virescens* and *S. frugiperda* were similar to those in *T. ni*: addition of B.t. protoxins known to be toxic to the insect resulted in DAPI incorporation into columnar epithelial cells in wholemounts. This assay method should prove useful with other economically important insects, including those which are difficult to rear in the laboratory (*e.g.* insects which burrow into stems and fruits).

*M. sexta* wholemounts posed problems not encountered with noctuids: although wholemounts were easily produced, staining reactions on untreated tissue were very different. The fluorochromes AO, PI and DAPI all entered columnar epithelial cells in the absence of B.t. toxins. *M. sexta* was laboratory-reared on both artificial diet and tomato plants. It was the only member of Sphingidae used in this assay and it is not known if all sphingids react to vital dyes this way or if dye uptake is peculiar to columnar cells of *M. sexta*. Since many researchers use *M. sexta* as their model in the study of B.t. toxin efficacy and mode of action, it would be beneficial to adapt this midgut model for

use in that insect.

Development of this model for use on other insects involves several factors. Larval size is the major constraint although wholemounts were produced here from second instar *T. ni* larvae. Successful wholemount preparation may require adjustment of enzyme incubation time, or even addition of other enzymes (*i.e.* collagenase). Knowledge of haemolymph composition is required to formulate suitable tissue bathing medium. Experiments to determine cell membrane permeability to vital dyes must be done before treatments can be applied to wholemount tissues. Evaluation of other fluorochromes may reveal more suitable indicators of membrane permeability. Specific probes may provide additional use for wholemounts, especially if used in conjunction with microspectro-fluorometric techniques.

### 5.5.2 Additional research on B.t. toxicity using midgut epithelial wholemounts

A curious toxicity effect was revealed here: Cry1B and Cry1D caused damage to cell membranes in basal (regenerative or undifferentiated) cells but not in fully differentiated columnar cells. This indicated that receptors present on undifferentiated cells were dissimilar from those on differentiated cells *i.e.* receptors changed over the cell's lifetime. It is natural to speculate that they will also change over the age of the insect (between instars). This may account partially for differential toxicity results demonstrated between instars (*i.e.* McGaughey, 1978). Also receptors may be expressed only during moulting. It may be possible to duplicate this response by adding juvenile hormone and/or 20-hydroxyecdysone (20-HT) to epithelial wholemounts prepared from intermoult larvae. Addition of *Lymantria dispar* fat body plus 20-HT to a primary midgut

cell culture from *M. sexta* supported stem cell proliferation and differentiation and resulted in cell cycling (Sadrud-Din *et al.*, 1994).

Alteration or elimination of known receptors may help clarify differences in binding. For example, addition of Cry1Ac to wholemounts treated previously with phosphotidylinositol-specific phospholipase C (PIPLC) should reveal whether receptors for Cry1Ac are bound to the membrane with a GPI anchor. Alteration in membrane permeability as a result of Cry1Ac binding after cleaving the GPI anchor would indicate presence of another receptor for Cry1Ac or non-specific binding. Likewise wholemounts provide an alternate method to test binding and pore formation in midgut epithelium after amino acid deletion or substitution experiments are done on B.t. toxins to determine specificity domains (Lu *et al.*, 1994). Wholemounts would be especially useful for insects for which cell lines are not available.

Laboratory selection for resistance is used to study binding characteristics for specific toxins. By using a carefully timed series of dissections from each generation of selected insects it may be possible to demonstrate exactly when receptors no longer bind toxin (or at least when pore formation stops). It might be possible to pinpoint the exact time in the cell cycle when resistance develops, and to determine if the primary function of the receptor has changed (*i.e.* if enzyme is no longer able to bind substrate).

### 5.5.3 Use of wholemounts for other research purposes

This epithelial tissue model should be useful for research on other aspects of midgut physiology including peritrophic membrane and basal lamina formation, cell cycling during moulting, midgut pH, and ion transport. For example, I have demonstrated that

peritrophic membrane is produced by epithelial tissue wholemounts. Production of PM components occurred soon after wholemount production and continued over time until a discrete film was formed. Comparison of PM formed by wholemounts with that from a whole insect would demonstrate which components are endogenous, and what, if any, additional hormonal or nutritional requirements are necessary. This technique provides the first means by which PM formation can be observed directly. Since PM is an important barrier against invading microbes, any agent that alters its structure or composition to facilitate pathogen entry would be a valuable tool in a biological control program. This technique provides a quick and easy method to screen such potential agents.

The epithelial model presented here might also be used for ion transport studies. Because wholemount tissue is essentially one layer thick and very fragile, it is not possible to physically separate fluids on apical and basal surfaces. But specific probes used in conjunction with quantitative measuring equipment should provide information on membrane permeability and function. With additional nutritional inputs and optimization of oxygen requirements it may be possible to develop this short-term tissue model into a viable organ culture.

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**Appendix 1 Hypotheses proposed to explain action of B.t. on midgut**

Angus (1968) found that *B. thuringiensis* var. *sotto* poisoning in *Bombyx mori* is similar to effects of valinomycin ingestion: larvae stop feeding, develop paralysis, and have increased haemolymph alkalinity. His hypothesis was that crystals solubilized in the gut are cleaved by digestive enzymes to yield a toxic peptide or polypeptide which acts on the membrane of midgut epithelial cells, affecting their selective permeability. He also noted that as valinomycin was a $K^+$ transporting agent, δ-endotoxin may also function as a $K^+$ transporter.

Travers *et al.* (1976) studied effects of *B. thuringiensis* var. *kurstaki* δ-endotoxin on mitochondria isolated from *B. mori* midgut epithelium to determine effects on cellular respiration and energy production. They found that δ-endotoxin had a similar effect as 2,6-dibromophenol, a known uncoupler of oxidative phosphorylation in mitochondria. In their proposed mechanism, crystal protein breakdown is mediated by alkaline conditions in the midgut. Toxic proteins and smaller toxic peptides, released with the aid of proteolytic enzymes, stimulate oxygen uptake, resulting in reduced levels of ATP production. A high demand for reducing potential by this increased $O_2$ uptake in a non-conservative electron transport system then increases the demand in the insect's catabolic processing of glucose (which explains the sudden influx of glucose into affected cells). The loss of ATP production caused by the uncoupling of oxidative phosphorylation also results in cessation of ATP-requiring processes such as glucose uptake by midgut epithelial cells. Without glucose, cell energy levels fall, paralysis begins, and osmotic integrity is lost. Travers *et al.* (1976) believe that disruption of ion transport at the cell

membrane is secondary to the uncoupling of oxidative phosphorylation.

English and Cantley (1985, 1986) showed that B.t. inhibited a vanadate-sensitive component of $^{86}Rb^+$ uptake (a good indicator of $K^+$ transport) into CHE cells (a *Manduca sexta* embryonic cell line that is capable of producing chitin). This suggests δ-endotoxin inhibits an active $K^+$ transport system. They further determined that B.t.k. inhibits purified dog kidney $Na^+,K^+$-ATPase and the site of action is on the cytoplasmic side of the membrane in human erythrocytes. In other words, δ-endotoxin must enter the cell before it can inhibit the $Na^+$ pump. Others (*i.e.* Fast *et al.*, 1978; Harvey and Wolfersberger, 1979; Sacchi *et al.*, 1986) have shown that the site of action is on the external surface of the cell membrane. English and Cantley also found the toxic component to be trypsin-sensitive.

Both Griego *et al.* (1979) and Harvey and Wolfersberger (1979) found that B.t. affected the $K^+$ permeability barrier of the insect membrane. Griego *et al.* (1979) reported inhibition of short-circuit current and potential by B.t. HD-1 toxin as early as 1 h after ingestion in *M. sexta*. Harvey and Wolfersberger (1979) added B.t. (pre-incubated in pH 11) to isolated *M. sexta* midgut and found that δ-endotoxin stimulated $O_2$ uptake and inhibited a specific component of active $K^+$-transport. In their model, B.t. causes irreversible inhibition of the electrogenic potassium pump across the plasma membrane: it stimulates efflux but has no effect on influx, therefore it must act on an active ($O_2$-dependent) component of the $K^+$ efflux. They also found it 100 times more effective when applied on the lumen side of the midgut than on the haemolymph side, and concluded that δ-endotoxin acts on the apical membrane.

Nickerson and Schnell (1983) compared the toxicity of cyclic peptides such as valinomycin, gramicidin S and polymixin B (known ionophores) to *B. thuringiensis* var. *israelensis* crystals on larvae of *Aedes aegypti,* and found similar signs of toxicity. They concluded that the mode of both lepidopteran- or dipteran-specific strains are similar, in that they modify the ion transport properties of larval midgut.

Gupta *et al.* (1985) found that the first action of B.t.k. (within 1 min) on the short-circuit current in isolated *M. sexta* midgut is on goblet cells, especially the contents of the goblet cavity. Secondary effects include increased blood $K^+$ and decreased blood $H^+$ concentration and a loss of potential difference *in vivo*. This led to changes in cell membrane potentials resulting in rapid changes in intracellular pH and $Ca^{2+}$, leading to a breakdown in cytoskeletal structure. They postulated that B.t.k. disrupts $K^+$ transport function in goblet cells by "perturbing the boundary lipid environment of the $K^+$ pump". They speculated that the goblet cell apical membrane contains a high density of B.t.k. binding sites, and the loss of $K^+$ transport is associated with a decrease in $K^+$ concentration in the goblet cell cavity. Their conclusion was that the crystal toxin is a specific inhibitor of the $Na^+$-independent $K^+$ pump.

Sacchi *et al.* (1986) found that B.t.k. toxin increased the permeability of BBMVs from *Pieris brassicae* midgut cells to $K^+$ but not to $H^+$ or $Na^+$. They determined the luminal side of the membrane to be the site of action in insect BBMVs, but B.t.k. does not increase membrane permeability in mammalian small intestine BBMVs.

Himeno *et al.* (1985) found that δ-endotoxin from *B. thuringiensis* var. *aizawai* caused swelling and lysis of TN-368 cells (a cell line derived from *T. ni* adult ovary) and

that cell swelling is dependent on the $Na^+$ or $K^+$ concentrations in the external medium. They proposed that B.t. toxins react with a receptor in the cell membrane and stimulate the action of $Na^+$ channels or ATPase. B.t.k induces a cascade of biochemical events ($Na^+$ influx through $Na^+$ channels, $K^+$ efflux through $K^+$-leak channels, and stimulation of the $Na^+/K^+$ pump by $Na^+/K^+$-ATPase in the cell membrane) resulting in a net influx of $Na^+$ and $K^+$ which causes cells to swell and burst.

Two theories closely substantiate cellular reactions to B.t. toxin activity (Tanada and Kaya, 1993). Knowles and Ellar (1987) proposed a two-step model in which toxin binds specifically to a receptor in the columnar cell plasma membrane and generates small pores in the membrane. These pores are formed either by toxins inserting into the membrane or by perturbing the resident plasma membrane molecules. Equilibration of ions through this pore results in a net influx of ions accompanied by water which causes swelling and lysis (the colloid osmotic lysis theory). As ions equilibrate through membrane pores, the electrogenic $K^+$ pump is inhibited, resulting in formation of a pH gradient.

The other hypothesis, based on Harvey and Wolfersberger (1979) and Harvey *et al.* (1983) is that the K pump, located on the apical ends of columnar epithelial cells, is irreversibly inhibited by B.t. δ-endotoxin, and the inhibition prevents movement of ions in the cell and leads to cytolysis.

**Appendix 1.1 Literature cited**

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**Appendix 2** Sterile artificial haemolymph solution (SAHS) modified from Palli and Locke, 1987. (Insect Biochem. 17:561-572)

| | g/l |
|---|---|
| KCl | 1.87 |
| $Na_2HPO_4$ | 0.71 |
| $MgCl_2.6H_2O$ | 2.03 |
| $CaCl_2$ | 0.44 |
| alanine | 0.50 |
| glutamic acid | 0.37 |
| glycine | 2.26 |
| histidine | 2.03 |
| lysine | 1.04 |
| serine | 1.90 |
| threonine | 0.26 |
| glucose | 0.898 |
| trehalose | 10.26 |

Osmolarity (measured): 232 mOs/kg

Adjust to pH 7.1 with NaOH. Filter sterilize using 0.22 µm filter.

Add 4.8 ml Antibiotic-Antimycotic (GIBCO) and 1.6 ml Ampicillin per litre bathing medium.

Antibiotic-Antimycotic (GIBCO):

- 10,000 units/ml penicillin G sodium (= 0.03 mg/ml)
- 10,000 µg/ml streptomycin sulfate (= 0.048 mg/ml)
- 25 µg/ml amphotericin B (= 0.12 µg/ml)

## Appendix 3 The use of trypan blue as a vital dye

Baron von Gleichen first invented vital colouring to study the uptake of particles into ciliates nearly two centuries ago, and Ranvier was the first histologist to adopt the method in 1875 (von Gleichen, 1778, and Ranvier, 1875, cited in Baker, 1958). Coloured substances were used in two ways to study living cells. Many different cell types allow certain dyes to infiltrate and colour certain pre-existent cellular components (Baker, 1958). Dyes used include dahlia, brilliant cresyl blue, Nile blue A, azures A and B, methylene blue, neutral red, Janus green, and Bismarck brown. These dyes simply diffuse into receptive cells.

Other cell types, specifically excretory and phagocytic cells, actively take up coloured particles during their regular cell function (Evans and Schulemann, 1914). The primary function of these cells is the engulfment of particles whose dimensions fall within certain physical limits. This type of vital dyeing is therefore a physical, not chemical, response. The particles need not be dyes, but they must be small enough to be taken in by single cells. The only dyes that are suitable in solution are those that tend to flocculate into particles of colloidal dimensions *(eg.* certain diazo dyes).

In 1906, Nicolle and Mesnil found that trypan blue is a vital dye when injected into healthy animals (Evans and Schulemann, 1914). Trypan blue is a diazo dye that exists as large molecules or ions. It has a tendency to clump, and is therefore captured once inside the cell. Whether soluble dyes or insoluble particles are used, pre-existent objects in the cells do not take up the colour; cellular or intracellular matter takes on only a slight diffuse colour; cells actively aggregate the particles into new, microscopically-visible

droplets or patches. Those particles and dyes that exist as small molecules or ions with little tendency to clump together are not used in this type of vital dyeing because they diffuse out of the cells as easily as they enter. Trypan blue does not diffuse into receptive cells (like neutral red does), but diffuses readily into the protoplasm and nucleus of dead or injured cells (Evans and Schulemann, 1914), so it is ideal for dead cell assays. Dye exclusion does not, however, prove cell viability: cell membranes are still selective even when cells are no longer capable of growth or multiplication (Tennant, 1964).

**Appendix 3.1 Literature cited**

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## Appendix 4 Production of *Bacillus thuringiensis* toxins

### Appendix 4.1 Production of Cry1Ac in *E. coli*

Cultures were grown in LB broth containing 100 µg ampicillin/ml for 2-3 days at 37°C (Ausubel *et al.*, 1992). Cells were harvested by centrifugation at 7,500 x g for 25 min at 4°C, washed in 0.1 M sodium phosphate pH 6.0, and passed twice through a French pressure cell at 11,000 psi. The resulting pellets were collected by centrifugation and washed through two centrifugations with 0.1 M sodium phosphate containing 0.1 M NaCl. Inclusion bodies were purified using either Renografin or NaBr discontinuous gradients. Pellets were resuspended in sterile water and mixed 1:1.8 with Renografin and centrifuged at 30,000 x ***g*** in an SW 28 rotor for 1 h (Masson *et al.*, 1989), or layered onto discontinuous gradients composed of 36% - 30% - 26% w/w NaBr and centrifuged at 52,000 x ***g*** in an SW rotor for 1 h (Moar *et al.*, 1989). The top gelatinous layer was discarded and the supernatant (containing inclusion bodies) was carefully removed from the pellet. Supernatant was diluted with sterile water and inclusion bodies were harvested by centrifugation at 10,000 x ***g*** for 15 min. Pellets were washed several times in sterile water to remove gradient materials. Purified inclusion bodies were solubilized in 50 mM sodium carbonate (adjusted to pH 9.5 with 0.1 N HCl) plus 10 mM dithiothreitol (DTT) for 60 min at 37°C (Knowles and Ellar, 1988). Protoxin production was confirmed by SDS-PAGE; protein concentrations were determined by the Bradford assay using BSA as a standard.

LB broth: (Ausubel *et al.*, 1992)

| | |
|---|---|
| bactotryptone | 10 g |
| bacto yeast extract | 5 g |
| NaCl | 10 g |
| distilled water | 1 l |

Adjust pH to 7.0 with NaOH; autoclave for 20 min.

**Appendix 4.2 Production of Cry1C-LM (cloned into a *cry*⁻ *Bacillus thuringiensis* strain)**

Cultures were grown on LB agar containing erythromycin (25 μg/ml) for 24 days at 28°C (by this time cells had lysed, releasing crystals and spores). Crystals and spores were harvested by scraping the plate surface with a sterile rubber policeman and washing the plates with sterile water. A portion of this crude preparation was solubilized in $Na_2CO_3$-HCl /DTT solubilization buffer at pH 9.5 for 60 min at 37°C.

**Appendix 4.3 Production of Cry1E (cloned into a *cry*⁻ *Bacillus thuringiensis* strain)**

Cultures were grown as described for Cry1C. Crystals and spores were harvested by scraping the plate surface with a sterile rubber policeman and washing the plates with sterile water. This aqueous solution was put into a separatory funnel and shaken vigorously for several minutes, after which liquid was decanted (modified from Pendleton and Morrison, 1966). Froth (containing spores) was then removed from the funnel, and the liquid re-added. The procedure was repeated several times, pooling 'froth' and liquid samples. The liquid sample was applied to discontinuous 36% - 30% - 26% NaBr gradients and centrifuged for 1 h at 20,000 x *g* at 4°C. A clear layer formed at the top was discarded; a dense layer just above the bottom pellet and the bottom pellet were retained and twice washed with water through a centrifugation at 10,000 rpm for 20 min at 4°C. The pellet fraction was discarded; the dense layer contained mostly crystals with a few spores and cells; and bottom pellet containing mostly cells was discarded. The crystal fraction was retained for use in midgut epithelial tissue wholemount assays and feeding bioassays. Protein concentration was determined using the Bradford method with BSA as the standard (6.271 mg/ml).

**Appendix 4.4 Production and purification of Cry3A protein from *E. coli* MV1190 cells** (protocol from C. Charlton, pers. comm.).

*E. coli* cells containing Cry3A were plated on LB agar containing ampicillin (75 µg/ml). Flasks containing 250 ml terrific broth containing ampicillin (75 µg/ml) were inoculated with *E. coli* cells and cultures were grown for 3 days at 37°C with shaking (300 rpm). Cells were pelleted at 5000 rpm for 10 min at 4°C. Pellets were resuspended in 14 ml 10 mM Tris - 10 mM EDTA pH 8.0. Cells were lysed by passage through a French pressure cell. Crystals were pelleted at 8000 rpm for 10 min at 4°C. Pellets were washed three times in cold 0.5 M NaCl - 0.1 mM EDTA, and once with 10 mM Tris - 0.1 mM EDTA. Purified crystals were solubilized for 2 h at 37°C in 3 ml 3.3 M NaBr and 0.1 mM EDTA with occasional mixing. Insoluble material was pelleted by centrifugation at 10,000 rpm for 10 min at room temperature, and the supernatant was recovered and stored at 4°C. Solubilized toxin was dialysed against 20 mM Tris buffer pH 8.0 at 4°C with 4 changes of buffer; purified protein was harvested from the dialysis tubing. Protein determinations were done using the Bradford assay.

Terrific Broth: (J. Sambrook *et al.*, 1989).

| | |
|---|---|
| bactotryptone | 6 g |
| bacto yeast extract | 12 g |
| glycerol | 2 ml |
| distilled water | 450 ml |

Autoclave for 20 min; cool to 60°C and add

| | |
|---|---|
| $KH_2PO_4$ | 2.31 g |
| $K_2HPO_4$ | 12.54 g |

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**Appendix 5. Key: DAPI staining in epithelium tissue wholemounts treated with various B.t. toxins.**

**Toxin form:**

| | |
|---|---|
| cry | insoluble crystal inclusions |
| sol | solubilized crystal proteins = protoxin |

**Stage:**

| | |
|---|---|
| F | intermoult larvae, feeding |
| M-> | moulting larvae |

**Cell type:**

| | |
|---|---|
| td | overall tissue damage |
| bas | small basal cells, presumably regenerative |
| sm | small columnar epithelial cells |
| med | medium-sized columnar epithelial cells |
| lrg | large columnar epithelial cells |
| ext | extruding columnar epithelial cells |

**Staining:**

| | |
|---|---|
| 4 | all cells (100%) |
| 3 | most cells (75%) |
| 2 | many cells (50%) |
| 1 | some cells (<25%) |
| r | cells on ridges |
| e | cells in areas along tissue edge |
| n | cells in nest-like pattern |
| c | cells in circular pattern |
| b | staining observed on basal side of tissue |
| p | pale DAPI staining |

** These may be used in combination:

| | |
|---|---|
| 2r | many cells on ridges |
| re3 | ridges and edges with most cells stained |
| 3r,2 | most cells on ridges stained; many cells stained |
| 1,r4 | few cells stained; all cells on ridges stained |
| 1,4b | few cells stained; all cells stained when tissue viewed from basal side |
| 2r,2 | many cells on ridges stained; many cells stained in remaining tissue |
| 1,4b | some cells stained; all cells stained when viewed from the basal side |

**Appendix 5.1.1 DAPI staining in midgut epithelium tissue wholemounts treated with Cry1Ac-LM crystals or protoxin.**

| Toxin form | Protein added (µg) | Stage | Instar | Time of day tissue isolated | Time tissue isolated post-moult | Final observation time post-treatment | Anterior | | | | | | Middle | | | | | | Posterior | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext |
| cry | | F | 4 | 15:20 | | 5 h 58 min | | | 1 | | 4 | | | | 1 | | 4 | | | | 1 | | 4 | |
| sol | 0.29 | F | 4 | 10:30 | | 26 h 27 min | | | 4 | | | 3 | | | 2 | | | 3 | | | 4 | | 3 | 3 |
| sol | 0.29 | F | 4 | 10:44 | | 14 h 45 min | | | 3 | | 3 | 3 | | | 3 | | 3 | 3 | | | 3 | | 3 | 3 |
| sol | 0.29 | F | 4 | 11:00 | | 23 h 30 min | | | | 1 | 1 | 1 | r1 | | e1 | e1 | | | | | | e1 | e1 | |
| sol | 0.29 | F | 4 | 11:11 | | 6 h 52 min | | 2 | | 3 | 2 | 2 | | | 2 | | 2 | 2 | | | 4 | | 4 | 2 |
| sol | 2.90 | F | 2 | 10:47 | 16 h 30 min | 3 h 5 min | | | | 3 | | | | | | 4 | | 1 | | | | 3 | | 1 |
| sol | 2.90 | F | 2 | 11:05 | 16 h 35 min | 3 h 9 min | | | | 4 | 2 | 2 | | | | 4 | 4 | 4 | | | | 4 | 4 | 2 |
| sol | 0.97 | F | 3 | 15:40 | 18 h | 5 h 23 min | | | | 4 | 4 | | | | | 4 | 4 | | | | | | 1,e4 | |
| sol | | F | 3? | 15:48 | | 5 h 20 min | | | | | | | | | | | | | | | | | | |
| sol | 0.48 | F | 3 | 11:48 | 19 h 46 min | 1 h 30 min | | | | | | 2 | | | | | | 2 | | | | | 4 | 1 |
| sol | 0.48 | F | 3 | 12:00 | 19 h 57 min | 5 h 37 min | | | 4 | | | 2 | | 2 | | | | 2 | | | | | | 1 |
| sol | 0.48 | F | 3 | 12:11 | 20 h 5 min | 5 h 37 min | | | 2 | | 2 | 3 | | 1 | | | | 1 | | | 1 | | | 1 |
| sol | 0.48 | F | 3 | 12:21 | 20 h 16 min | 5 h 16 min | | | 2 | | 2 | 2 | | | 3 | | 2 | 2 | | | 2 | | 4 | 1 |
| sol | 0.58 | F | 3 | 12:46 | 42 h 10 min | 4 h 58 min | | | | 4 | | 1 | | | | 4 | | 1 | | | | 4 | | 1 |
| sol | 0.58 | F | 3 | 12:57 | 42 h 20 min | 4 h 55 min | | | | 4 | | 2 | | | | 2 | | | | | | 4 | | 1 |
| sol | | M-> | 4 | 11:15 | | 8 h 5 min | 4 | | | | | | | | 4 | | 4 | | | | 4 | | 4 | |
| sol | 0.58 | M-> | 4 | 11:25 | | 44 min | | | | | | 1 | | | | | | 1 | | | | | | 1 |
| sol | | M-> | 4 | 11:30 | | 7 h 55 min | 4 | 3 | | e1 | e1 | | 4 | 3 | | e1 | e1 | | 4 | 3 | | e1 | e1 | |
| sol | 0.10 | M-> | 4 | 12:20 | | 6 h 55 min | | 4 | | | 4 | 4 | | 4 | | | 4 | 4 | | 4 | | | 4 | 4 |
| sol | 2.90 | M-> | 4 | 12:52 | | 3 h 54 min | | | 2 | 3 | | | | | 1 | 4 | | 2 | | | 3 | 4 | | 1 |
| sol | | M-> | 4 | 13:00 | | 1 h 45 min | | 1 | 4 | | 4 | 4 | | | 4 | | 4 | 4 | | | 4 | | 4 | 4 |
| sol | 2.90 | M-> | 4 | 13:06 | | 3 h 48 min | | | | 4 | | 2 | | | | 3 | | 2 | | | 1 | 4 | | 3 |
| sol | 2.90 | M-> | 4 | 14:08 | | 17 min | | 4 | | | 4 | | | 4 | | | 4 | | | 4 | | | 4 | |
| sol | 2.90 | M-> | 4 | 14:15 | | 2 h 13 min | | 4 | | | 4 | | | 4 | | | 4 | | | 4 | | | 4 | |
| sol | 0.97 | M-> | 4 | 15:24 | | 5 h 35 min | | 4 | | | 4 | | | 4 | | 4 | 4 | | | 4 | | | | |
| sol | 0.00 | M-> | 4 | 15:30 | | 5 h 30 min | | 3r | | | 3r | | | e1 | | | | | | | | | | |
| sol | 19.88 | M-> | 4 | 17:14 | | 2 h 55 min | | | | 4 | 4 | | | | | 4 | 4 | | | | | 4 | 4 | |
| sol | 19.88 | M-> | 4 | 17:30 | | 2 h 43 min | | | 4 | | 4 | | | | 4 | | 4 | | | | 4 | | 4 | |

**Appendix 5.1.2 DAPI staining in midgut epithelium tissue wholemounts treated with Cry1Ac-KH protoxin.**

| Toxin form | Protein added (µg) | Stage | Instar | Time of day tissue isolated | Time tissue isolated post-moult | Final observation time post-treatment | Anterior | | | | | | Middle | | | | | | Posterior | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext |
| sol | 0.64 | M-> | 4 | 10:20 | 40 h 17 min | 3 h 35 min | 3 | 4,n,c | | | 4 | 4 | | 2b | | | 3p | | | 3b | | | 3 | 3 |
| sol | 0.64 | M-> | 4 | 10:30 | 40 h 25 min | 3 h 30 min | | 1b | | | 2 | 4 | | | | | 4 | | | | | | 4 | 3 |
| sol | 0.64 | M-> | 4 | 10:37 | 40 h 34 min | 3 h 25 min | | 1b | | | 4 | | | 1b | | | 3 | | | 1b | | | 4 | 4 |

**Appendix 5.2 DAPI staining in midgut epithelium tissue wholemounts treated with Cry1Ab protoxin.**

| Toxin form | Protein added (µg) | Stage | Instar | Time of day tissue isolated | Time tissue isolated post-moult | Final observation time post-treatment | Anterior | | | | | | Middle | | | | | | Posterior | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext |
| sol | 0.55 | F | 4 | 16:10 | 20 h | 3 h 21 min | | 3p | | | 3 | | | | | | 2 | | | | | | 2 | |
| sol | 0.55 | F | 4 | 16:20 | 20 h | 3 h 20 min | | | | | 2 | | 4 | 2 | | | 3 | | | | | | 1 | |
| sol | 1.11 | F | 4 | 14:53 | | 3 h 24 min | | | | 3 | | 3 | | | | 3 | | 3 | | | | | | 1 |
| sol | 1.11 | F | 4 | 15:04 | | 3 h 17 min | | | | 3 | | | | | | 3 | | | 1 | | | | | 1 |
| sol | 1.11 | F | 4 | 15:15 | | 3 h 14 min | | | | 3 | | 1 | | | | 2,r | | 1 | | | | 2,r | | 2 |
| sol | 2.77 | F | 4 | 15:22 | | 3 h 8 min | | | 3p | | 4 | 4 | | | | | 3 | 4 | | | | 1 | | 1 |
| sol | 2.77 | F | 4 | 15:30 | | 3 h 5 min | | | 2 | | 4 | | | | 3 | | 3 | | 1 | | | | | 1 |
| sol | 2.77 | F | 4 | 15:40 | | 2 h 59 min | 3 | | | 3 | | | | | | 3 | | 3 | 2 | | | 2 | | 2 |
| sol | 0.55 | M-> | 4 | 09:53 | 40 h 5 min | 3 h 45 min | | 1b | | 3r,2 | | | | | | 3 | | | | | | | 2 | 1 |
| sol | 0.55 | M-> | 4 | 10:10 | 40 h 11 min | 3 h 34 min | | 1 | | | 1r | 2 | | | | 2 | | 1 | | | | 2 | | 1 |
| sol | 0.55 | M-> | 4 | 10:55 | 40 h 42 min | 3 h 15 min | | 4c | | | 2 | 2 | | | | | 3 | | | 4,1n | | | 1 | 3 |
| sol | 0.55 | M-> | 3 | 11:45 | | 5 h 12 min | | 4,n | | | | | | 4,n | | | 3p | | | 4 | | | | |
| sol | 0.55 | M-> | 3 | 11:45 | | 8 h 5 min | | 4 | | | 4 | | | 4 | | | 4 | | | 4 | | | r1 | 1 |
| sol | 0.55 | M-> | 3 | 11:55 | | 2 h 44 min | | 3 | | | r1 | | | 4,n | | | | | | 4,n | | | | |
| sol | 0.55 | M-> | 4 | 15:47 | | 3 h 35 min | | 4 | | | 3 | | | 4 | | | 3 | | | 4 | | | | 1 |
| sol | 0.55 | M-> | 4 | 15:55 | | 3 h 33 min | | 4 | | | 4 | | | 4 | | | 4 | | | 4 | | | | |
| sol | 1.11 | M-> | 4 | 09:35 | 38 h 43 min | 3 h 53 min | | 4 | | | 2r | 1 | | 4 | | | 2 | | | 4 | | | 1r | 1 |
| sol | 1.11 | M-> | 4 | 09:55 | 38 h 53 min | 3 h 45 min | | 4 | | | | 2 | | 4 | | | 2 | 4 | | 4,n | | | 3 | 4 |
| sol | 1.11 | M-> | 4 | 11:20 | 40 h 35 min | 3 h 50 min | | 1 | | | 4 | | | 1,4b | | | 4 | | | 2,2n | | | 3 | |
| sol | 1.11 | M-> | 4 | 11:30 | 40 h 40 min | 3 h 45 min | | 4,n | | | 4 | | | 2 | | | 3 | | | 4,n | | | 2 | 3 |
| sol | 1.11 | M-> | 4 | 13:54 | | 5 h 5 min | | | | | 3 | | | | | | | 2 | | | | 2 | 3 | 3 |
| sol | 1.11 | M-> | 4 | 14:05 | | 4 h 58 min | | 1,4b | | | 4 | | | 4b | 2 | | 2 | | | | 2 | | 2 | |
| sol | 1.11 | M-> | 4 | 14:15 | | 4 h 55 min | | 4,n | | | 4 | | 4 | 4,n | | | 4 | | | 4,n | | | | 1 |
| sol | 1.11 | M-> | 4 | 14:21 | | 4 h 53 min | | 4 | | | 4 | | | 4b | | | 4 | | | 4 | | | | 1 |
| sol | 1.11 | M-> | 3 | 14:40 | | 4 h 38 min | | | 3 | | 3 | | | | 3 | | 3 | | | n | | | | 1 |
| sol | 1.11 | M-> | 3 | 15:04 | | 2 h 55 min | | 4 | | | 3 | | | 4 | | | 1 | | | 4 | | | 1 | |
| sol | 1.11 | M-> | 3 | 15:15 | | 3 h | | 3,n | | | | | | 1n | | | | | | n | | | 1 | |
| sol | 2.77 | M-> | 4 | 10:05 | 39 h | 3 h 45 min | | 4 | | | 3 | 4 | | 4,n | | | 4 | 4 | | 4,1n | | | 3 | 4 |
| sol | 2.77 | M-> | 4 | 10:15 | 39 h 08 min | 3 h 40 min | | 4,n | | | 4 | 4 | | | | | 2 | 4 | | 4,2n | | | 2 | 3 |
| sol | 2.77 | M-> | 4 | 11:38 | 40 h 47 min | 3 h 35 min | | 1 | | | 4 | | | | | | 4 | | | 1 | | | 3 | |
| sol | 2.77 | M-> | 4 | 11:50 | 40 h 55 min | 3 h 25 min | | 4,n | | | 4 | | | 4b | | | 4 | | | 4,n | | | 4 | |
| sol | 2.77 | M-> | 4 | 12:25 | 39 h 42 min | 7 h 10 min | | | | | 3 | | | | | | 4 | | | | | | 4 | |
| sol | 2.77 | M-> | 4 | 12:40 | 39 h 50 min | 6 h 55 min | 4 | | | 4 | | | | 1 | | 4 | | | | 1b | | 4 | | |
| sol | 2.77 | M-> | 4 | 12:50 | 39 h 58 min | 6 h 40 min | | 4c | | | 4 | | | 4 | | | 4 | | | 4c | | | 4 | |
| sol | 2.77 | M-> | 4 | 12:55 | 40 h 7 min | 6 h 35 min | 4 | 4 | | | 4 | | 4 | 4 | | | 4 | | | 4c | | | 4 | |
| sol | 2.77 | M-> | 4 | 13:15 | 40 h 20 min | 6 h 10 min | 4 | | | | | | 4 | | | | | | | 1b | | | | 4 |
| sol | 2.77 | M-> | 4 | 16:52 | 43 h 35 min | 2 h 18 min | | 4 | | | 4 | | | 4b | | | 4 | | | 4 | | | 2 | 4 |
| sol | 2.77 | M-> | 4 | 17:03 | 43 h 43 min | 2 h 17 min | | 4 | | | 4 | | | 3b | | | 4 | | | 4b | | | 4 | |
| sol | 2.77 | M-> | 4 | 17:13 | 43 h 49 min | 2 h 5 min | | 4b | | | 4 | | | 4b | | | 4 | | | 2b | | | 1 | 1 |

**Appendix 5.3 DAPI staining in midgut epithelium tissue wholemounts treated with Cry1B protoxin.**

| Toxin form | Protein added (µg) | Stage | Instar | Time of day tissue isolated | Time tissue isolated post-moult | Final observation time post-treatment | Anterior | | | | | | Middle | | | | | | Posterior | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext |
| sol | 0.65 | F | 4 | 18:25 | | 2 h 52 min | | 1,1n | | | | 1 | | | | | | 1 | | | | | | 1 |
| sol | 1.30 | F | 3 | 15:03 | 20 h 44 min | 3 h 44 min | | | | 1,3r | | 1 | | | | 1,3r | | 1 | | | | 2,3r | | 2 |
| sol | 1.30 | F | 3 | 15:15 | 20 h 50 min | 3 h 43 min | | | | 2,3r | | | | | | 2,3r | | | | 1b | | 2,3r | | 1 |
| sol | 3.26 | F | 3 | 14:10 | | 3 h 14 min | | | | r1 | | | | | | r1 | | 1 | | | | | | 1 |
| sol | 3.26 | F | 3 | 14:20 | | 3 h 6 min | | | | r1 | | 1 | | 1n | | r1 | | 1 | | | | | | 1 |
| sol | 3.26 | F | 4 | 18:00 | | 2 h 59 min | | | | | | 1 | | 2r | | | | 3 | | | | | | |
| sol | 0.65 | M-> | 3 | 11:55 | | 5 h 10 min | | 1n | | | r2 | | | | | | r1 | | | n | | | | |
| sol | 0.65 | M-> | 4 | 18:20 | | 2 h 53 min | | 4 | | | | | | 4 | | | | | | 4 | | | | |
| sol | 0.65 | M-> | 4 | 18:40 | | 2 h 38 min | | 1n | | 3r | | 2 | | 2,1n | | 2,3r | | | | 1,1n | | | | 2 |
| sol | 3.26 | M-> | 4 | 18:10 | | 2 h 54 min | | 3 | | | | 3 | | 4,n | | | 2,3r | | | 4,n | | | | |
| sol | 3.26 | M-> | 4 | 18:15 | | 2 h 53 min | | 4,n | | | | 4 | | 4,n | | | 2r | 4 | | 4 | | | | |

**Appendix 5.4.1 DAPI staining in midgut epithelium tissue wholemounts treated with Cry1C-LM crystals or protoxin.**

| Toxin form | Protein added (µg) | Stage | Instar | Time of day tissue isolated | Time tissue isolated post-moult | Final observation time post-treatment | Anterior | | | | | | Middle | | | | | | Posterior | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext |
| cry | | M-> | 4 | 11:36 | | 4 h 47 min | | | | | | | | | | | | | | | | | | |
| sol | | M-> | 4 | 16:25 | | 4 h 40 min | 1 | | | | | | 1 | | | 1 | 2 | | 1 | | | | | |

**Appendix 5.4.2 DAPI staining in midgut epithelium tissue wholemounts treated with Cry1C-KH protoxin.**

| Toxin form | Protein added (µg) | Stage | Instar | Time of day tissue isolated | Time tissue isolated post-moult | Final observation time post-treatment | Anterior | | | | | | Middle | | | | | | Posterior | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext |
| sol | 0.80 | F | 3 | 12:00 | | 3 h 9 min | | | 3 | | 3 | | | | | | | | | | 3 | | 3 | 2 |
| sol | 0.80 | F | 3 | 12:07 | | 3 h 22 min | | | | | 2 | | | | | | 2e | | | | 3 | | 2 | 1 |
| sol | 0.80 | F | 3 | 12:12 | | 3 h 22 min | | | | | 1 | | | | 1 | | 1 | | | | 3 | | 3 | 1 |
| sol | 1.59 | F | 3 | 15:20 | 20 h 58 min | 3 h 44 min | | | 2 | 2,2r | | | | | | 1,2r | | 1 | | | | | | 1 |
| sol | 1.59 | F | 3 | 15:34 | 21 h 9 min | 3 h 35 min | | | | 2,3r | | 2 | | | | 2r | | | | | | | | |
| sol | 3.98 | F | 3 | 11:45 | | 3 h 34 min | | | 2 | | 2 | | | | 3r | | 3 | | | | | | 4 | |
| sol | 3.98 | F | 3 | 11:48 | | 3 h 35 min | | | | | 3 | | | | | | 2 | | | | | | 4 | |
| sol | 3.98 | F | 3 | 11:54 | | 3 h 30 min | | | 2 | | 3 | | | | 2r | | 2r | | | | 2 | | 4 | 1 |
| sol | 3.98 | F | 3 | 14:45 | | 2 h 53 min | | | | 4 | | | | | | | | 1 | | | | 1 | | 1 |
| sol | 3.98 | F | 3 | 14:50 | | 2 h 57 min | | | | 2p | | | | | | 3r | | | | | | | | |
| sol | 0.80 | M-> | 3 | 12:10 | | 5 h 31 min | | | | | 1 | | | | | | 1 | | | | | | 1 | |

**Appendix 5.5 DAPI staining in midgut epithelium tissue wholemounts treated with Cry1D protoxin.**

| Toxin form | Protein added (µg) | Stage | Instar | Time of day tissue isolated | Time tissue isolated post-moult | Final observation time post-treatment | Anterior | | | | | | Middle | | | | | | Posterior | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext |
| sol | 1.40 | F | 3 | 15:40 | 21 h 20 min | 3 h 32 min | | 3b | | | | 1 | | 3b | | 1r | | | | 3b | | | | |
| sol | 1.40 | F | 3 | 15:46 | 21 h 30 min | 3 h 33 min | | 1b | | 3r | | | | 1b | | 3r | | | | 3b | | | | 1 |
| sol | 3.51 | F | 3 | 14:30 | | 3 h 1 min | | | | r1 | | | | | | r1 | | | | | | | | |
| sol | 3.51 | F | 3 | 14:40 | | 2 h 56 min | | | | | | | | | | r1 | | | | | | | | |
| sol | 3.51 | F | 4 | 15:30 | | 5 h 22 min | | 3p,c | | | | | | 4c | | | | | | 4c | | | | |
| sol | 0.70 | M-> | 3 | 12:05 | | 5 h 35 min | | 4c | | | | | | 4 | | | | | | 4 | | | | |
| sol | 0.70 | M-> | 4 | 14:45 | | 3 h 48 min | | 4 | | | | 1 | | 4 | | | | | | 4 | | | | 1 |
| sol | 0.70 | M-> | 4 | 15:05 | | 3 h 47 min | | 4 | | | | 1 | | 4 | | | | 1 | | 4 | | | | |
| sol | 0.70 | M-> | 4 | 15:12 | | 3 h 42 min | | 4 | | | | | | 4 | | | | | | 4 | | | | |
| sol | 0.70 | M-> | 4 | 15:22 | | 3 h 37 min | | 4 | | | | | | 4 | | | | | | 4 | | | | |

**Appendix 5.6 DAPI staining in midgut epithelium tissue wholemounts treated with Cry1E crystals or protoxin.**

| Toxin form | Protein added (µg) | Stage | Instar | Time of day tissue isolated | Time tissue isolated post-moult | Final observation time post-treatment | Anterior | | | | | | Middle | | | | | | Posterior | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext |
| cry | 0.10 | F | 3 | 14:00 | | 7 h 20 min | | | | | | | | | 2r | | 2r | | | | | | | |
| cry | 0.31 | F | 4 | 14:56 | | 6 h 3 min | | | re3 | | re3 | | | | re3 | | re3 | | | | re3 | | re3 | |
| cry | 0.63 | F | 4 | 15:46 | | 12 h 2 min | | | | | | | | | r2 | | 2 | | | | | | 1 | |
| cry | 0.07 | F | 4 | 11:00 | 14 h | 7 h 25 min | | | | | | 1 | | | | | | 1 | | | | | | 1 |
| cry | 0.07 | M-> | 4 | 10:02 | | 8 h 5 min | | | | | | 3 | | | | | | 3 | | | | | | 1 |
| cry | 0.07 | M-> | 4 | 10:40 | | 7 h 42 min | | | | | | 2 | | | | | | 2 | | | | | | 2 |
| cry | 0.10 | M-> | 3 | 11:35 | | 23 h 30 min | | | | | | 1 | | | | | | 1 | | | | | | 1 |
| cry | 0.00 | M-> | 3 | 11:40 | | 23 h 45 min | | | | | | | | | | | | 2 | | | | | | |
| cry | | M-> | 3 | 11:45 | | 4 h 38 min | | | | | | | | | | | | | | | | | | |
| cry | 0.10 | M-> | 4 | 13:41 | | 7 h 22 min | | | | | | 1 | | | | | | 1 | | | | | | 1 |
| sol | 9.04 | F | 4 | 15:05 | | 4 h 30 min | | | 4 | 4 | | | | | 4 | 4 | | | | | 4 | 4 | | |
| sol | 9.04 | M-> | 4 | 11:47 | | 3 h 57 min | | 4 | | | 4 | | | 4 | | | 4 | | | 4 | | | 4 | |
| sol | 9.04 | M-> | 4 | 12:00 | | 3 h 50 min | | 3e | | | 3e | | | 3e | | | 3e | | | 3e | | | 3e | |
| sol | 9.04 | M-> | 4 | 12:10 | | 3 h 45 min | | 3e | | | 3e | | | 3e | | | 3e | | | 3e | | | 3e | |
| sol | 3.01 | M-> | 4 | 12:17 | | 23 h | 3 | | | | 3 | | 3 | | | | 3 | | 3 | | | | 3 | |
| sol | 3.01 | M-> | 4 | 14:35 | | 4 h 35 min | | | | | 4 | 1 | | 4 | | | 4 | 1 | | | | | | |
| sol | 0.00 | M-> | 4 | 14:53 | | 4 h 29 min | | 4 | | | 4 | | | 4 | | | 4 | | | 4 | | | 4 | |

**Appendix 5.7 DAPI staining in midgut epithelium tissue wholemounts treated with Cry2A crystals or protoxin.**

| Toxin form | Protein added (µg) | Stage | Instar | Time of day tissue isolated | Time tissue isolated post-moult | Final observation time post-treatment | Anterior | | | | | | Middle | | | | | | Posterior | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext |
| cry | 0.65 | F | 3 | 13:35 | | 2 h 50 min | | | | | | | | | | | | | | | | | | |
| cry | 2.61 | F | 3 | 10:50 | 15 h | 6 h 25 min | | | | | 1 | 1 | | | | | 1 | 1 | | | | | | |
| cry | 0.65 | F | 3 | 11:05 | 15 h | 6 h 20 min | 1 | | | | 1 | | | | | | | | | | | | | |
| cry | 2.61 | F | 3 | 11:17 | 15 h | 6 h 8 min | | | | | | | | | | | | | | | | | | |
| cry | 0.65 | F | 3 | 11:27 | 15 h | 5 h 58 min | | | | | | 1 | | | | | | 1 | | | | | | 1 |
| cry | 0.65 | F | 3 | 11:27 | 15 h | 3 h 13 min | | | | | | | | | | | | | | | | | | |
| cry | 0.65 | M-> | 4 | 13:30 | | 3 h | | | | | | | | | | | | | | | | | | |
| sol | 1.13 | M-> | 3 | 11:00 | | 4 h 20 min | | | 4 | | 4 | | | | 4 | | 4 | | | | 4 | | 4 | |
| sol | 1.13 | M-> | 3 | 11:07 | | 4 h 18 min | | | 4 | | 4 | | | | 4 | | 4 | | | | 4 | | 4 | |
| sol | 1.13 | M-> | 3 | 16:15 | | 2 h 5 min | 1 | | 3 | | 4 | 4 | 1 | | 3 | | 3 | 4 | 1 | | 3 | | 4 | 4 |
| sol | 1.13 | M-> | 3 | 16:30 | | 3 h 20 min | 4 | | e4 | | e4 | | 4 | | e4 | | e4 | | 4 | | e4 | | e4 | |

**Appendix 5.8 DAPI staining in midgut epithelium tissue wholemounts treated with Cry3A protoxin.**

| Toxin form | Protein added (µg) | Stage | Instar | Time of day tissue isolated | Time tissue isolated post-moult | Final observation time post-treatment | Anterior | | | | | | Middle | | | | | | Posterior | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext | td | bas | sm | med | lrg | ext |
| sol | 15.82 | F | 4 | 11:25 | 16 h | 8 h 3 min | | | | | | | | | | 1r | | 1 | | | | | | 1 |
| sol | 21.10 | F | 4 | 11:45 | 16 h | 7 h 45 min | | | | | | 1 | | | | | | 1 | | | | | | 1 |
| sol | 21.10 | F | 4 | 11:53 | 16 h | 7 h 37 min | | | | 1r | | 1 | | | | 1r | | 1 | | | | | | 1 |
| sol | 15.82 | F | 4 | 12:00 | 16 h | 7 h 30 min | | | | | | 1 | | | | | 2 | | | | | | 3 | |
| sol | 21.10 | F | 4 | 12:05 | 16 h | 7 h 25 min | | | | | | | | | | | 2r | 2 | | | | | 2r | 2 |
| sol | 10.55 | F | 4 | 12:10 | 17 h 30 min | 3 h 50 min | | | | | | | | | | | | | | | | | | |
| sol | 10.55 | F | 4 | 12:20 | 17 h 30 min | 3 h 40 min | | | | | 1r | 1 | | | | | 1r | 1 | | | | | | 1 |
| sol | 15.82 | F | 4 | 12:27 | 17 h 30 min | 3 h 38 min | | | | | | 1 | | | | | | 1 | | | | | | 1 |
| sol | 10.55 | F | 4 | 12:45 | 17 h 30 min | 3 h 25 min | | | | | | 1 | | | | | | 1 | | | | | | 1 |
| sol | 10.55 | F | 4 | 13:34 | 17 h 30 min | 3 h 31 min | | | | | 1r | | | | | | 1r | | | | | | | |
| sol | 10.55 | F | 3 | 12:26 | 42 h | 1 h 20 min | | | | | | 1 | | | | | | 1 | | | | | | 1 |
| sol | 0.11 | M-> | 4 | 11:25 | | 5 h 49 min | | | | | | 2 | | | | | | 2 | | | | | | 1 |
| sol | 0.11 | M-> | 4 | 11:38 | | 5 h 37 min | | | | | | 1 | | | | | | 1 | | | | | | 1 |
| sol | 0.11 | M-> | 4 | 11:45 | | 5 h 35 min | | | | | | 1 | | | | | | 1 | | | | | | 1 |
| sol | 0.11 | M-> | 4 | 11:50 | | 5 h 29 min | | | | | | 1 | | | | | | 1 | | | | | | 1 |
| sol | 0.11 | M-> | 4 | 12:05 | | 5 h 20 min | | | | | | | | | | | | | | | | | | |